TOWARDS UNDERSTANDING COAGULATION SYSTEM DERANGEMENTS IN TRAUMA

Imran Raza

Centre for Trauma and Neuroscience

The Blizard Institute

Barts and The London School of Medicine and Dentistry

Queen Mary, University of London

Submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy

Queen Mary University of London

2016

Abstract

Bleeding following trauma is exacerbated by coagulopathy and is a major cause for morbidity and mortality worldwide. The mechanisms influencing coagulopathy are not clear and require further exploration. A large pragmatic trial showed survival benefit from use of antifibrinolytics, suggesting fibrinolysis as a major component of coagulopathy. The overall aim of this work is to examine the incidence of fibrinolysis and magnitude of its activation in trauma, the protein mechanisms that may dominate in its modulation and to explore whether other coagulopathies may be important in trauma.

A prospective cohort study of 325 injured trauma patients was carried out. Bloods samples were collected on arrival to ED and data collected on injury characteristics, demographics, blood component therapy and outcomes. An extension to the study included over 1500 patients recruited from 6 European centres in the same manner.

Only 5% of patients showed evidence of ROTEM Lysis, however, 59% of patients had evidence of fibrinolytic activation (FA) in that they had PAP levels over 1500 mcg/L. FA correlated with severity of head, chest and extremity trauma. Having FA conferred a significantly higher chance of needing blood or blood products, having septic complications and longer hospital stay as well as significantly increased mortality compared to those with no FA.

High levels of PAI-1 were found to be associated with lower levels of FA even when tPA was high. High levels of circulating TAFI or TAFIa were not associated with reduced markers of fibrinolysis suggesting they don't play a role in modulation. FXI depletion was associated with higher FA but its mechanism of action is unclear.

Hierarchical clustering was successfully used to classify 1229 patients with a biological dataset of 21 variables into 6 distinct groups. These showed predominantly fibrinolytic patterns of coagulation derangement. One very small group showed signs of consumptive coagulopathy.

Acknowledgements

﴿I begin in the name of Allah, The Beneficent, The Merciful﴾

I would like to thank my parents who have always supported and nurtured my endeavours and my sense of inquisitiveness and curiosity throughout my childhood, education and medical career.

My wife has been the back bone of both my career and research. This thesis is as much hers as mine. Doing this while bringing up 2 wonderful children who from the earliest age grew up with the words “PhD and Prof” in their vocabulary.

I cannot thank Professor Karim Brohi enough for his support, encouragement and above all patience and continued backing throughout this time. I have been honoured to be one of his students. I can only hope that I have done some fraction of a justice to the potential he saw in me originally to take on this project.

My friends in the TCAU made this research experience a wonderful and all the more fruitful one. Our support for each other has undoubtedly led to our success and achievements in academia and one would hope life. Sirat, Ross, Claire, Dan, Henry, Joanna, Anita, Elaine, Karen, Zane, Simon, Andy, Veena, Jo and Cath to name but a few. I am indebted to you all.

The support of the department of discrete mathematics was invaluable in shaping the latter part of this work under the guidance of Professor Arrowsmith and Rui Carvalho.

Mike Walsh, who from my very early time as an SHO lit the spark of trauma in my mind.

Perhaps most importantly the patients recruited to ACITII that enabled this work to be carried out. Without their support and the support of their families none of this work would be possible. Those that suffered or even lost their life as a result of their injuries will have supported the valuable work of ACITII and its results that will change the way we see and manage the disease of trauma in the future.

Declaration of originality

I, Imran Raza, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

I accept that the College has the right to use plagiarism detection software to check the electronic version of the thesis.

I confirm that this thesis has not been previously submitted for the award of a degree by this or any other university.

Signature:

Date: 28.02.2016

Publications directly related to this work

Raza I, Davenport R, Rourke C, Platton S, Manson J, Spoors C, Khan S, De_ath HD, Allard S, Hart DP, Pasi KJ, Hunt BJ, Stanworth S, MacCallum PK, Brohi K. The incidence and magnitude of fibrinolytic activation in trauma patients**. Journal of Thrombosis and Haemostasis 2013**; 11:307–14.

Funding

Imran Raza received partial funding by way of a studentship grant provided through the Engineering and physical research council (EPSRC).

Grant reference EP/K50290X/1

EPSRC had no influence on the work carried out or the writing of this thesis.

Contents

List of Abbreviations

A2AP	α-2-antiplasmin
ACIT II	Activation of Coagulation and Inflation in Trauma II
APTT	Activated partial thromboplastin time
AIS	Abbreviated Injury Score
aPC	Activated Protein C
AT	Antithrombin
ATC	Acute Traumatic Coagulopathy
BD	Base Deficit
CA5	Clot Amplitude Measured at 5 minutes (ROTEM)
Cryo	Cryoprecipitate
D&I	Demographic & Injury
DIC	Disseminated Intravascular Coagulation
ED	Emergency Department
ELISA	Enzyme Linked Immunoabsorbent Assay
ELT	Euglobulin lysis time
FA	Fibrinolytic activation
FBC	Full Blood Count
FFP	Fresh Frozen Plasma
Fg	Fibrinogen
GBD	Global Burden of Disease
GCS	Glasgow Coma Score
HF	Hyperfibrinolysis
HTS	Hypertonic Saline
ICU	Intensive Care Unit
INR	International Normalised Ratio
ISS	Injury Severity Score
ISTH	International Society of Thrombosis and Haemostasis
IV	Intravenous

LAR	Legally Appointed Representative
LOS	Length of Stay
MOF	Multi Organ Failure
P100	Proteomics
PAI-1	Plasminogen activator inhibitor-1
PAP	Plasmin-antiplasmin complex
PC	Protein C
PCI	Potato carboxipeptidase inhibitor
PF1+2	Prothrombin Fragments 1 and 2
PLAR	Professional legal appointed representative
POC	Point of Care
PRBC	Packed Red Blood Cell
PS	Free Protein S Antigen
PT	Prothrombin time
PTr	Prothrombin time ratio
PTT	Partial Thromboplastin Time
RBC	Red Blood Cell
RCT	Randomised Controlled Trial
RF	Resuscitation Fluids
RLH	Royal London Hospital
ROTEM	Rotational Thromboelastometry
RRT	Renal Replacement Therapy
SBP	Systolic Blood Pressure
SD	Standard Deviation
SFMCs	Soluble Fibrin Monomer Complexes
SOP	Standard Operating Procedure
TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TAFIa	Activated Thrombin Activatable Fibrinolysis Inhibitor
TARN	Trauma Audit and Research Network
TCAU	Trauma Clinical Academic Unit

tF	Tissue Factor
TIC	Trauma Induced Coagulopathy
TM	Thrombomodulin
tPA	Tissue Plasminogen Activator
TXA	Tranexamic Acid
U&E	Urea and Electrolytes
VCTs	Viscoelastic Tests
VTE	Venous Thromboembolism
VWF:Ag	Von Willebrand factor antigen
YLL	Years of life lost

Table of Figures

List of Tables

Chapter 1

Introduction

1.1 Global burden of trauma

Trauma is an important cause of mortality worldwide. It is estimated that, globally, 4.8 million people died due to “injury”, in the year 2013(1). This included transport injuries as well as accidental and non-accidental injury. Although this is receding in some parts of the world, traumatic injuries still contribute as a significant proportion of mortalities worldwide. This is particularly the case in the younger population. Amongst the age groups 15-19 and 20-24, mortality from transport injuries alone formed the single highest proportion of deaths in 1990 and in 2010 amongst males(2). The global burden of disease study (GBD) also found that “road injury” was the eighth highest cause of death over all. This is important as it seems to affect younger age groups. Globally, trauma injuries represented the eighth highest cause of years of life lost (YLL), and YLL could be argued to be an even more significant measure than mortality alone.

1.2 Trauma in a regional context

In the United Kingdom in 2014, trauma related deaths were amongst the leading causes of mortality in those aged less than 50 years old(3), which reflects the global trend described in para 1.1 above. Since mortality from trauma predominantly affects younger age groups it also contributes significantly to loss of productivity in society. The YLL measure alone cannot relay the total human cost involved. Trauma will affect all socioeconomic backgrounds and families of those involved, a cost which may never be quantifiable. Those that survive traumatic injuries may suffer temporary or permanent disability, thereby contributing further to the personal and financial cost and productivity loss to society.

1.3 Temporal distribution of mortality in trauma

Deaths that occur in trauma appear to do so in a multi-modal distribution. These have been broadly classified historically into immediate, early and late deaths(4,5). Although some changes in timing and distribution of deaths has occurred since implementation of modern trauma systems, the overall causes and distribution are relatively unchanged(6).

Immediate deaths have been described as those occurring within an hour of injury. These are largely unavoidable deaths due to massive neurological or cardiac and great vessel injuries. Intervention strategies in this phase of mortality are centred on injury prevention and education.

Late deaths are those that occur days or weeks after injury. These are generally as a result of sepsis, multi-organ failure and may be influenced by comorbid conditions as well as age. Essentially this can be seen as late physiological collapse. Although there is a great deal of research in this area and some promising advances being made(7), much involving critical care specialists, it is a difficult and complex problem to address.

Early deaths, on the other hand, have broadly speaking been thought of as those that might be potentially avoidable with advances in trauma management. These could include improved pre-hospital recognition and management(8,9), better access to imaging of a higher quality and resolution(10), improved access to interventions such as surgery and interventional radiology and improved understanding and implementation of damage control resuscitation(11,12) and surgical protocols to name a few. These early deaths occur in the first few hours of injury. As these deaths have been labelled as possibly preventable, research has tended to focus on this phase of mortality to improve outcomes. The window to recognise and manage these patients early is small with some reports showing as little as a median of 1.6hrs from injury to death, and others showing mean time to death of about 6 hours (13–15).

1.4 Mode of death in trauma

Two modes of death appear to predominate worldwide in this population irrespective of timing or indeed differences in mechanisms regionally. Neurological injury accounts for the majority of deaths in trauma. Pang et al have reported that as many as 72% of deaths occur as a result of brain injury(16) with mean rates being between 44 and 64%(17). These deaths

tend to occur in all time scales following trauma but the majority occur immediately or early(14,18).

The second most common cause of death is haemorrhage or exsanguination. These historically accounted for up 40% of all deaths and around 50% of early deaths(18). In the context of modern trauma systems it is accepted that they account for up to between 15% and 28% of all trauma deaths(17). Exsanguination occurring as a result of heart or great vessel injury is, of course, most likely to be un-survivable. However, death due to exsanguination from sources other than those stated is deemed possibly preventable and therefore an important area of management that is always under scrutiny. Where bleeding is as a result of a single site of trauma or even defined penetrating injury, haemorrhage control may be, relatively speaking, achievable. Blunt trauma on the other hand poses a difficult challenge in achieving surgical control. Moreover, bleeding associated with blunt trauma can often be exacerbated by coagulopathy. Since this is 'non-compressible' bleeding, it makes it even more difficult to definitively control.

1.5 Bleeding and coagulopathy in trauma

Bleeding in trauma is compounded by coagulopathy. The term trauma induced coagulopathy (TIC) is given to coagulopathy that occurs after severe injury. This was historically thought to be due to a combination of dilution of clotting factors (from fluid or blood administration), consumption of factors, hypothermia and acidosis(19). These are all important mechanisms and certainly do contribute but appear to be only relevant in extremes of physiology(20) or perhaps in later stages of injury and resuscitation. The relevance of coagulopathy, however, cannot be underplayed. Where coagulopathy is present mortality is over three-fold higher(21,22) but importantly in this group multi-organ failure (MOF), intensive care unit (ICU) stay and overall hospital length of stay (LOS) are all also much higher(21) suggesting significant systemic derangements other than simply coagulopathy. Over the years further influences of TIC have been defined and begun to be

understood(Figure 1.1)(23). Of course like any disease or condition, co-morbidities and genetics will play a role in physiological response also, but these are outside our influence in emergent trauma management. In recent years the central area of trauma research has been acute traumatic coagulopathy (ATC).

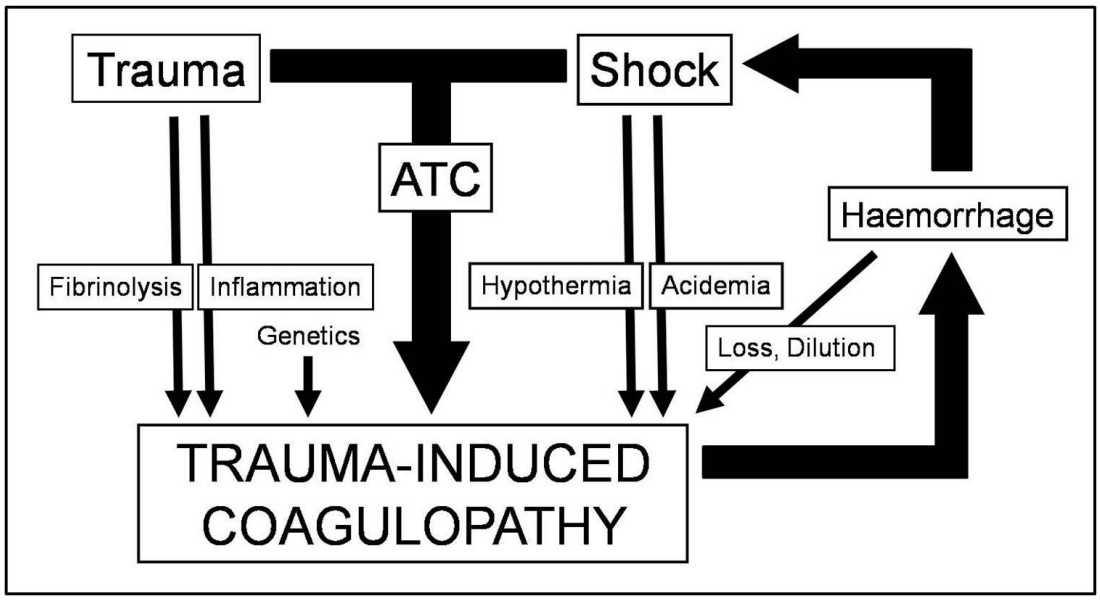


Figure 1.1 Drivers of trauma-induced coagulopathy(23)

1.6 Acute traumatic coagulopathy

The historical norms were challenged with the publication of the seminal paper by Brohi and colleagues in 2003(24). They showed that 25% of patients arriving in the emergency department (ED) already had established coagulopathy. Other studies have corroborated these results(21,22) leading to increased interest and research into ATC and its mechanisms. In the same study, notably, this coagulopathy was not associated with increased fluid administration (and therefore dilution). Those that arrived with coagulopathy had a four-fold increase in mortality, and where this coagulopathy occurred in the presence of more severe injuries, measured by the injury severity score (ISS), conferred between 80-100% mortality (for ISS≥45). The mechanism for this was postulated to be a systemic anticoagulation mediated through the protein-C (PC) pathway. Thrombin

was thought to be diverted to binding soluble thrombomodulin (TM) and away from its usual role in converting fibrinogen to fibrin. This 'thrombin switch' was tested in prospective trials to determine the relationships and mechanisms involved. Indeed Brohi and colleagues, in further work, showed PC was significantly reduced in the presence of TM(25), suggesting PC was being converted to activated protein-C (aPC). But this only occurred when there was evidence of significant hypoperfusion, as evidenced by base deficit (BD). In fact, even where there was thrombin generation, TM levels were only high when BD was high. Coagulation was also measured in this study based on prothrombin time (PT) and partial thromboplastin time (PTT). Coagulopathy only occurred in these severe hypoperfusion states. This showed that there was adequate thrombin generation in these states, but the combination of thrombin generation and hypoperfusion lead to the coagulopathy. The coagulopathy itself could be caused by aPC blocking the cleavage of factors V and VIII and curtailing further activation (Fig 1.2)(26).

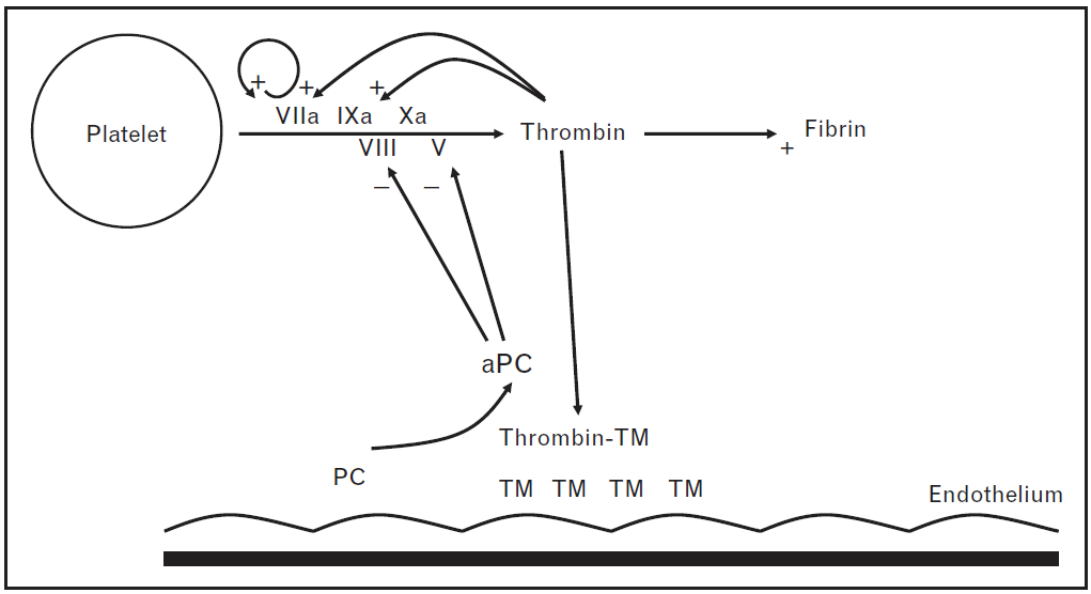


Figure 1.2 Thrombin-Thrombomodulin activating PC

Hyperfibrinolysis (HF) or pathological fibrinolysis has also been implicated in coagulopathy following trauma. D-Dimers levels have been shown to be raised significantly post trauma. This had previously been understood as disseminated intravascular coagulation (DIC)(27). Brohi et al also demonstrated rise in D-Dimer in those patients with low PC(25) certainly suggesting evidence for fibrinolysis. It has previously been described that aPC is an inhibitor of the control mechanisms for fibrinolysis mainly by inhibiting plasminogen activator inhibitor-1 (PAI-1)(28), and in certain conditions the affinity for aPC and PAI-1 increases dramatically(29). Indeed PAI-1 is reduced significantly with low PC levels in the above described situation also(25). This argument for a "thrombin switch" and PC mediated coagulopathy was consolidated when it was shown that PC levels fall as TM increases but also that fibrinogen levels are maintained with higher levels of TM(30). It was also demonstrated that tissue plasminogen activator (tPA) increases with worsening hypoperfusion but was also associated with lower PAI-1 levels. Evidence of fibrinolysis was demonstrated again with high D-Dimer in these patients. So as well as aPC curtailing coagulation upstream (Figure 1-2), it was also promoting fibrinolysis downstream by removing the inhibitors of plasminogen activation (Figure 1-3)(26). This coagulopathy and its clinical sequelae only occur in the presence of both tissue hypoperfusion and tissue injury(31). Until recently aPC could not be measured directly and so our assumptions have been based on the observations of PC. Cohen and colleagues(32) have further tested these hypotheses and developed an in house assay to measure aPC. Indeed, their work has shown that coagulopathy is only demonstrated in the presence of both hypoperfusion and injury corroborating earlier work by Frith and colleagues(31). This same subset of patients in their study showed high levels of aPC and low PC. Findings also confirmed low factor V and VIII activity in the presence of high aPC and indeed high levels of tPA and D-Dimer in the same patients, strengthening both parts of the hypothesised mechanism.

The summarised putative model is therefore as follows;

1. Hypoperfusion (shock) and tissue injury result in release of TM and tPA (from endothelium)
2. Thrombin is diverted to complex with TM, therefore also contributing to coagulopathy by not cleaving fibrinogen to make fibrin
3. Thrombin-TM complex catalyses the conversion of PC to aPC

Here two further possible mechanisms result in clinical coagulopathy;

a) aPC curtailing thrombin generation through inhibition of factors V and VIII
b) aPC inhibiting PAI-1 and therefore allowing the generated tPA to promote HF unchecked

Which of these mechanisms are more influential is still under debate. Certainly they are all potential targets for intervention and may indeed work synergistically to cause ATC and worsen TIC.

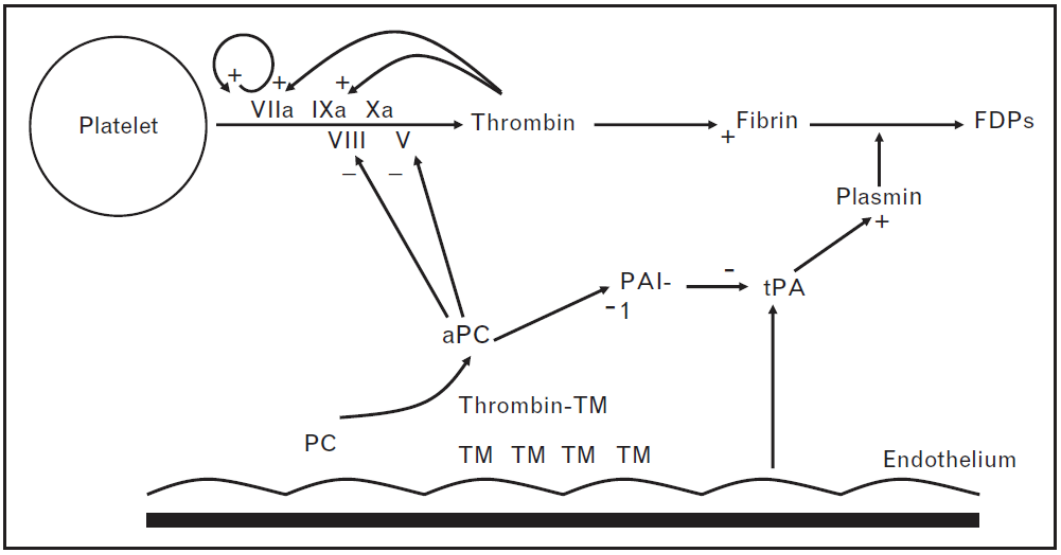


Figure 1.3 Release of fibrinolysis by inhibition of PAI-1

1.7 Hyperfibrinolysis

The Cochrane review of antifibrinolytics in elective surgery showed benefit by reduction of transfusion requirements(33) with no significant risk of venous thromboembolism (VTE). There is certainly some overlap in the insults which trauma patients suffer and elective surgery patients undergo during major surgery. This led to a large pragmatic randomised controlled trial (RCT) to assess the benefit of tranexamic acid (TXA) in trauma(34). The CRASH-2 trial found that there was significant reduction in relative risk death from all cause mortality as well as death from bleeding. On the face of it these data suggest that HF is a significant mechanism in ATC. After all, the previous work from various units has shown evidence of lysis as a phenotype in trauma patients. But the Crash-2 trial did raise some very important questions about HF.

1.8 Diagnosis of coagulopathy

An important issue that needs to be addressed in the current clinical practice is how we diagnose coagulopathy, particularly in the emergent setting. Once coagulopathy has been established its utility in managing therapy would then need review. The work mentioned to date has used PT and PTT to diagnose coagulopathy. Maegele and colleagues have also

used low platelet counts in conjunction with PT(21). These tests have been useful as markers of coagulation for researchers after fact. In clinical practice however they are of questionable use in the emergent trauma patient for a number of reasons. It is also important to note that they were originally developed for completely different utility(35,36). Historically the threshold for coagulopathy has been PT ratio (PTr) of greater than 1.5. This appears to be an arbitrary figure and a review carried out by Frith and colleagues of 3,646 cases from a European multicentre study showed that mortality was more likely above PTr of just 1.2(31). This was much lower than previously considered. In fact other parameters supported this threshold being considered a true coagulopathy. Red blood cell (RBC) and fresh frozen plasma (FFP) requirements were also significantly higher with PTr >1.2 (figure 1.4). This certainly leads us to rethink how we use PT in discussing coagulopathy. Rather than a high threshold of 1.5 there seems to be a dose dependant relationship with outcomes, i.e. the more coagulopathic trauma patients are on arrival the worse the likely outcomes.

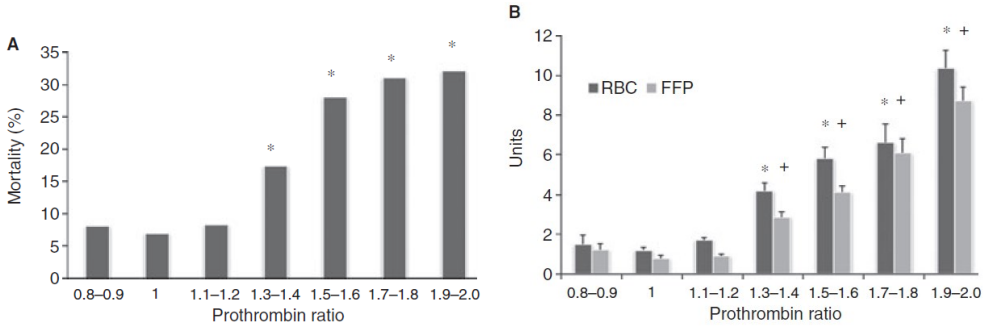


Figure 1.4 Dose dependant response in outcomes of PTr >1.2(31)

The threshold for PTr is important to appreciate but using PT as an adjunctive tool in resuscitation of exsanguinating acutely unwell trauma patients is still challenging. Davenport and colleagues(37) reported a median time of 78 minutes until the PTr was available to clinicians. In this cohort of 300 subjects the PTr became available in less than 30 minutes on only two occasions. This is clearly unacceptable as a tool in emergency care.

They went on to assess a hand held point of care (POC) device for measuring PTr at the bedside. In non-coagulopathic subjects the POC device agreed with the lab in 99% of cases. However in coagulopathic subjects there was significant difference between the POC device and the lab standard resulting in the POC device giving a high false negative rate making it unacceptable. The main focus on this work was to test ROTEM in its detection rate for ATC. They described a threshold of clot amplitude measured at 5 minutes (CA5) of <36mm correlated with PTr >1.2. Significantly this threshold also predicted patients receiving RBCs and FFP at significantly higher rates. This work now provides a tool that can diagnose coagulopathy within 5 mins and predict transfusion requirements. Other centres have used ROTEM to diagnose coagulopathy(38). But they have also taken it to the next step and use it to guide management of coagulopathy. Their algorithms are based more on experience however than on current evidence which is unfortunately lacking. Nevertheless, using ROTEM and other viscoelastic tests (VCTs) maybe more accurate and timely than relying on PTr and conventional lab assays.

1.8.1 Principles of viscoelastic tests to diagnose coagulopathy

Viscoelastic tests have been described as far back as 1948(39). In the past 15 years or so they have come back into favour as a research and a diagnostic tool in haemostasis. They are utilised to assess and follow coagulopathy and bleeding in cardiac surgery(40), obstetric haemorrhage(41), liver transplantation(42) and of course trauma(37,43,44) to name but a few disciplines. There are two major proprietary tests available; TEG (Haemonetics Corporation, Braintree, MA, USA) and ROTEM (TEM International, GmbH, Munich, Germany). ROTEM is much more commonly in use in Europe where as TEG is much more common in the USA. The principles are broadly similar. Both VCTs measure the 'elasticity' or the 'clot firmness' of the blood as it goes from its liquid form to its semi solid form during coagulation. They do this under low shear stress conditions to give a graphical representation of coagulation and following this of fibrinolysis. Blood is added to a cup and

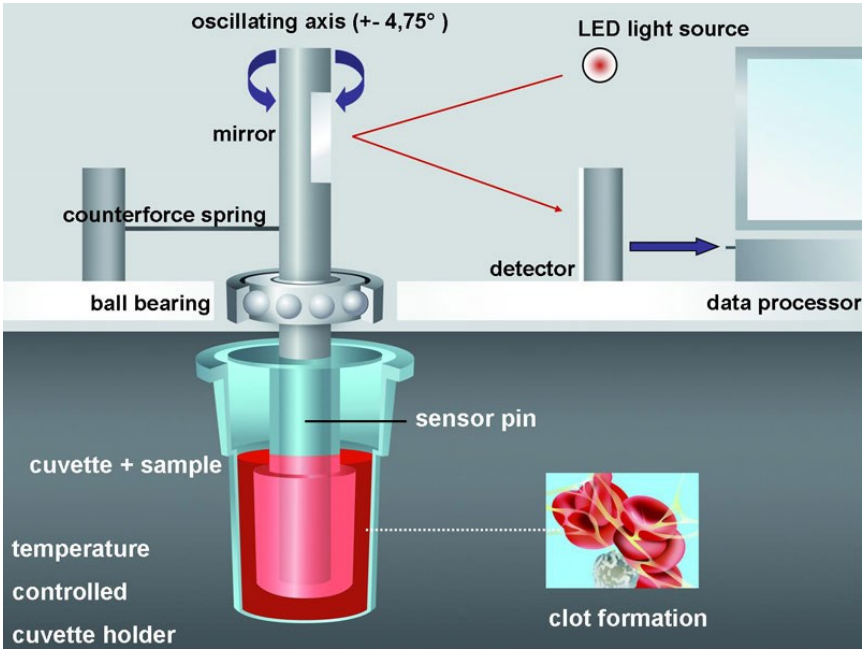


Figure 1.5 ROTEM Mechanism (reproduced from www.rotem.de)

activators are added to speed up the process of coagulation. The cup is then mounted into a pin mechanism. The pin (in the case of ROTEM) or the cup (in the case of TEG) oscillates in the machine. As a clot begins to form between the cup and pin the elastic properties of the blood change and the resistance between the cup and pin in increased. The machine is able to measure this resistance or impedance and give us a digital readout. ROTEM displays this as a 'thromboelastogram' on the screen and also quantifies this output in various measures (Figure ROTEM and Table 1). Given that the test is performed on whole blood the resulting trace represents the balance between clotting and fibrinolysis at all times throughout the clotting process. It also reflects the contribution of platelets in coagulation. A number of specific assays are available and are each used to look at specific aspects of the clotting system:

- **ExTEM** – Activated clotting via extrinsic pathway using tissue factor
- **InTEM** – Activated clotting via intrinsic pathway using ellagic acid
- **FibTEM** – Activated clotting via extrinsic pathway and platelets inhibited (with cytochalasin-D) to assess the contribution of fibrin polymerisation alone

- **ApTEM** – Activated clotting via extrinsic pathway with antifibrinolytic added to assess the level of fibrinolysis (by comparing ApTEM and ExTEM)

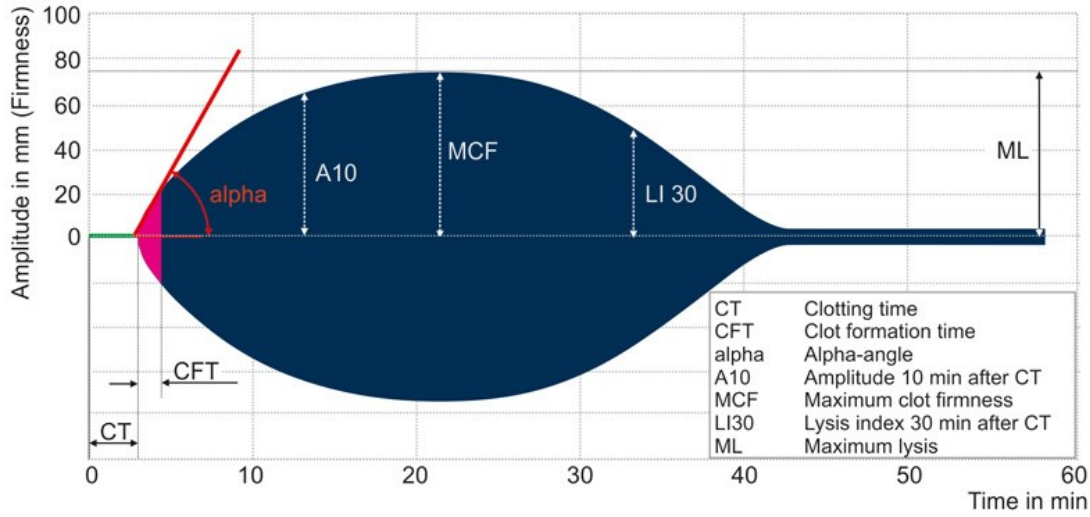


Figure 1.6 Example Thromboelastogram and measured values (reproduced from www.rotem.de)

Table 1-1 Description of ROTEM reported parameters

Standard nomenclature ROTEM parameters. (Modified from Tem International GmbH, Martin-Kollar-Strasse 13–15, 81829 München, Germany, 2014.)

ROTEM Parameter	Description	Interpretation of parameter
CT(seconds) Clotting time	Time from start of sample run to appearance of first detectable clot (amplitude of 2mm)	**Initiation phase** Lag-phase of thrombin generation (dependent on activity of coagulation factors)
CFT(seconds) Clot formation time	Time from CT until level of clot firmness reaches 20mm	**Amplification phase** Initial clot strengthening by formation of fibrin fibres
α-angle (degrees)	Kinetics of clot development - angle between centre line and tangent to the curve through the 2mm amplitude point	**Thrombin burst** Maximum velocity of clot formation (dependent on platelet function and fibrin polymerisation)
CA'x' (mm) Clot amplitude at time 'x'	Mechanical clot quality at fixed time point e.g. 10 minutes (CA10)	**Propagation phase** Clot strength at fixed time point determined by platelets, fibrinogen and Factor XIII
MCF (mm) Maximum Clot Firmness	Mechanical clot quality	Maximal clot strength – determined by platelets, fibrinogen and Factor XIII balanced by fibrinolysis
ML(%) Maximum Lysis	Clot amplitude at 90 minutes (max time) compared to MCF	**Clot termination** Determined by amount of fibrinolysis

The data acquired as a result of ROTEM can be potentially more useful than PT in an emergent setting for a number of reasons.

When measuring PT or PTT we get a single figure as a result. In ROTEM the course of clot formation or 'clot firmness' can be followed until a designated time, which potentially gives us a lot more data. This, in theory, could be much more useful (but also more difficult to interpret). ROTEM could also be used as a true point of care test if correctly implemented. For example, at our institution a ROTEM machine is in the resuscitation bay in ED. A sample could be processed as soon as blood is available. Although ROTEM can be used to follow clotting activity for up to eight hours in a research setting, useful information could be derived within minutes of commencing analysis. This is of course much faster than lab PT in the real world(37). In addition, the information derived using the ExTEM and FibTEM tests in conjunction could be used to tailor blood component therapy(45). It should be stressed, however, that this specific practice is still awaiting validation and further trial evidence. A recent consensus conference (46) failed to arrive at firm conclusions on this point and were unable to recommend specific product therapy based on VCT parameters alone.

1.9 Scope for further research

Accepting Davenport's work(37) the coagulopathy described thus far has been diagnosed on the basis of PT and PTT. These are tests that were designed historically for a completely different utility(35,36). They certainly are not directed at detecting fibrinolysis for example. Tests for fibrinolysis, such as euglobulin lysis time (ELT), do exist but these are generally difficult to carry out and need to be done in a closely controlled lab environment(47). Certainly they are not suitable for rapid evaluation of HF in an emergent setting or indeed in the presence of antifibrinolytics(48). VCTs such as ROTEM are still relatively rare in day to day clinical use in EDs. In some settings they have been used to detect HF by measuring the degree of lysis on ROTEM. A few small studies have published this data but these would suggest that the incidence of HF (measured by ROTEM) is extremely low. In fact it ranges

between 3% and 6% (43,49,50). This is in contrast to the incidence of coagulopathy measured by PT or PTT which has been shown to be around 25% to 34%(21,22,24). However even in those who present with coagulopathy based on PT there is evidence of fibrinolytic activation (FA) as evidenced by D-Dimer and tPA (30). These previous findings would suggest that FA is more prevalent than those exhibiting HF alone. Furthermore, the documented mortality in patients with HF by ROTEM is 80-100% (43). This does not correlate with mortality described in other studies where coagulopathy found on PT is much lower. In addition CRASH-2 showed a survival benefit of 9% in groups receiving TXA compared to those that didn't. Again this is in contrast to the reports of HF by ROTEM which show that virtually nothing can reverse this coagulopathy and it inevitably leads to mortality. Despite the findings of CRASH-2 some centres still give antifibrinolytics only in response to ROTEM HF. CRASH-2 evidence would again suggest that FA is more prevalent than seen on ROTEM and these ROTEM-undetected cases would benefit from TXA.

The exact mechanisms of HF in trauma also need further review. The role of PAI-1, Thrombin activatable fibrinolysis inhibitor (TAFI) and α-2-Antiplasmin (A2AP) needs to be adequately understood. Their relative contributions in the modulation of the fibrinolysis would allow focussed research pathways and routes to possible intervention.

1.10 Aims of this body of work

The aims of the work presented here are therefore as follows:

1. To investigate the true prevalence of FA or HF in our trauma population
2. To determine the ability of ROTEM to detect FA and/or HF in our trauma population
3. To understand the relative contributions of the fibrinolysis pathway factors in modulation or control of fibrinolysis in trauma
4. Use all of the above to understand better the mechanisms responsible in development of ATC
5. To understand whether ATC is of a single type of coagulopathy or can encompass different coagulopathies

Chapter 2

Methods

2.1 Study Design and participants

This thesis comprises a discrete body of work nested within a much larger ongoing research project originally based at the Royal London Hospital (Whitechapel, London, UK) (RLH) and The John Radcliffe Hospital (Oxford, UK). The 'Activation of Coagulation and Inflammation in Trauma II' (ACIT II) study originally received ethical approval in November 2007 from the East London and City Regional Ethics committee (07/Q0603/29) to recruit 500 patients. In June 2010 this was extended to allow 2800 patients in total. In 2013 the study was extended to 5000 subjects to be recruited from European and US partners. The European Community funded FP7 study entitled "Targeted Action for Curing Trauma Induced Coagulopathy" (TACTIC; project ID F3-2013-602771) now incorporates 6 European trauma receiving hospitals in Amsterdam, Cologne, Copenhagen, Oslo, London & Oxford.

The study itself is an ongoing prospective observational cohort trial of trauma patients admitted to the above centres. I was part of the admission and recruitment team for the RLH. Patients were recruited on admission to the ED and followed up to discharge or day 28 of admission. Blood samples were drawn on admission and analysed for various markers of coagulation as well as ROTEM. Data were collected on injury characteristics, patient demographics and admission physiology. Fluids and blood or blood products administered in the first 24 hours were noted. Specific Injury assessment was completed for each patient by our in house data collectors and scored based on abbreviated injury scores (AIS) categories which were used to give total Injury severity scores (ISS). For the first 28 days of admission, data were collected on organ injury, ventilation requirement, critical care stay and various other outcomes.

2.1.1 Inclusion criteria

All patients arriving in the ED that triggered trauma team activation were eligible for inclusion. The criteria at the RLH are as follows:

- GCS<14 or RR<10 or >29 or Systolic BP<90

- Chest trauma with altered physiology
- Person hit by train
- Amputation proximal to wrist or ankle
- Occupant ejected from vehicle
- Fatality in same vehicle as occupant
- Suspected pelvic fracture
- Person trapped under vehicle
- Suspected open or depressed skull fracture
- Fall from >2 metres
- Polytrauma with burns
- Penetrating trauma (neck to groin or proximal to elbow/knee)
- Explosions
- Industrial accidents

2.1.2 Exclusion criteria

Exclusion criteria for recruiting patients were as detailed below. The majority of these were set up so that we only captured patients who would have ATC and no other extrinsic or acquired forms of pre-existing coagulopathy:

- Age <16 years
- Patients transferred from other hospitals
- Patients who were already incarcerated prior to the presenting incident
- Patients presenting more than two hours after time of injury
- Patients who received more than 2,000 ml of intravenous fluids prior to arrival in the ED
- Patients with burns >5% of their total body surface area
- Patients taking anticoagulant medication other than aspirin
- Patients with a known bleeding diathesis
- Patients with moderate to severe liver disease (Child-Pugh classification B or C)

2.2 Consent process

Recruiting patients to this particular project proved an interesting challenge. Our interest in ATC means that we had to obtain blood samples at the earliest possible time following injury and not more than two hours afterwards. Severely injured trauma patients often

display a variable level of consciousness or are even unconscious and possibly intubated and ventilated (for the most severe injuries). Those that are awake and seem alert are nevertheless likely to be in a difficult state of mind following a significant psychologically challenging event as well as physical trauma. They may be in significant pain or discomfort. For these and other reasons they may not be in a position to have a useful conversation about medical trials and be able to understand the minutiae of the trial or the consent process. Indeed it may be totally inappropriate to approach this subject at the time. Nevertheless, in order to be able to gain useful information blood samples are required immediately on arrival at the hospital. Our ethics approval therefore allows us to recruit the subject into the trial and obtain blood samples with the agreement of the admitting trauma team leader. This is usually the ED consultant but could also be the registrar. They are the 'professional legal appointed representative' (PLAR) for each patient at the time of admission. All the ED consultants were well versed on the protocol and study itself beforehand and were extremely supportive. Once the PLAR had given approval we could take blood samples and collect data as required based on our protocol. After this at the earliest possible stage (usually the next morning) if the patient is conscious and capable of having a conversation we would approach them about the trial and have a discussion as required. If they were then in a position to understand, they may then give or withhold consent to enter the trial. If they were unable to have a conversation or communicate clearly at this stage we would return daily until they were in a position to do so. For those patients that were very unwell and admitted to critical care requiring sedation or ventilation the relatives or next of kin were approached at the earliest possible time. In the context they are the 'legally appointed representative' (LAR). They are able to give or withhold assent on the patient's behalf. Even if assent was obtained from the LAR we would still return on a regular basis to reassess the suitability for the patient themselves to give consent. In the event that the patient did not have capacity to give or withhold

consent and no LAR was forthcoming, Police and social service teams would attempt to look for relatives or next of kin on a daily basis until found. All these attempts whether successful or otherwise to obtain consent were logged in our consent database. All PLAR consents were copied into the patient notes and when consent or assent was completed by the patient or LAR these were also noted in medical notes.

An overwhelming majority of patients and LARs agreed with no issues. This was predictable as this is an observational trial and, apart from having blood tests drawn, does not affect care in any way. At times in the department we were running other interventional trials also. If this was the case consent for both trials would be sought simultaneously. In the event that consent was withdrawn for the interventional trial, again, the vast majority of patients still gave consent for this observational cohort study.

2.3 Blood sampling

Blood samples were drawn on patient arrival in the ED (within in 20 minutes). The samples taken on admission were labelled '0' hour samples. These were taken as the baseline samples. Following this, samples were also taken at 24 and 72 hours. In addition, for patients requiring blood transfusion, further samples were also taken following 4, 8 and 12 units of red blood cells (RBCs). ACIT II was set up to investigate all aspects of TIC and outcomes in trauma. My work focusses on ATC and therefore I have only utilised the 0 hour sample. Therefore, I will hereafter discuss only the 0 hour sampling protocol. It should be noted, however, that the other time point or transfusion point sampling was carried out in a virtually identical manner with only minor changes.

On arrival in the ED a blood sample was sent urgently for the clinical care and decision making of the patient. This included arterial or venous blood gas, full blood count (FBC), clotting screen, fibrinogen level, urea and electrolytes (U&E) and group and screen. In addition to this sample the research team member (myself or one of 5 other team

members on the research rota) would obtain a further 20-25 millilitres (mls) of blood from an easily accessible vessel. This was usually from the antecubital fossa or from a femoral vein. This sample was immediately placed into various sample vacutainers as per Table 1.

Table 2-1 Summary of blood sample collection patient arrival in ED

Type of vacutainer	Blood Volume (mls)	Reagent used as preservative	Intended use
Coagulation	4.5 x2 vials	0.109 M buffered sodium citrate (3.2%)	Coagulation proteins
Coagulation	2.7 x 1 vial	0.109 M buffered sodium citrate (3.2%)	ROTEM
Proteomics (P100)	6.5 x 1 vial	Protease inhibitor	Assay of unstable or novel proteins
PAXgene (RNA)	2.5 x 1 vial	RNA stabiliser	RNA analysis (gene expression)

2.4 Sample processing and storage

All the samples were processed immediately on arrival in our near patient research lab in the trauma clinical academic unit (TCAU). This was located in the hospital itself and within 5 minutes walk from the ED. Before 2012 the ROTEM samples were processed in the TCAU with the other samples. After 2012 a ROTEM machine was acquired by the ED and ROTEM samples were processed in the resuscitation bay in ED itself before processing other samples in the TCAU lab. The samples were processed by myself and the other research

fellows. We all underwent a period of training and shadowing more experienced research fellows before we were allowed to process samples without supervision in order to maintain consistent techniques and familiarise ourselves with the protocol. From 2013 when the study was extended to our European collaborators, our standard operating procedures (SOP) were shared with them. Many of their research fellows came to London for a periods of training and shadowing in order to familiarise themselves with our protocol and to accurately duplicate it at their centres in order to maintain consistency and quality.

2.4.1 Coagulation samples

The 4.5ml coagulation tubes were centrifuged at 1760g for 10 minutes in a Clinispin Horizon 853VES Laboratory Centrifuge (Woodley Equipment Company Ltd, Bolton, UK). The top 2/3 of the plasma was removed from each tube and returned to the centrifuge for a further 10 minutes at 1760g. From the remaining single spun samples the top 0.5mls was aliquoted into a 0.6ml Cliklok microcentrifuge tube (Simport, Jencons, UK). The "buffy coat" and top layer of red cells were aliquoted into a 1.5ml Fisherbrand microcentrifuge tube (Fisher Scientific, UK). The original 2/3 of plasma, once double spun, was again aliquoted in 0.5ml increments into 3 x 0.6 ml Cliklok microcentrifuge tubes. All of these microcentrifuge tube were labelled and barcoded and stored at -80^{o}C in partitioned cardboard cryoboxes

2.4.2 Proteomics P100 samples

The P100 vacutainers were centrifuged using the same equipment at 2500g for 5 minutes to activate the mechanical filter. The resulting plasma was then aliquoted in 0.5ml samples into as many microcentrifuge vials as possible, usually between 6 and 8. All microcentrifuge tubes were labelled and barcoded and stored at -80^{o}C in partitioned cardboard cryoboxes.

2.4.3 PAXgene RNA

These samples required no processing. They were simply labelled and stored at -20^{o}C within 10 minutes of collection for 24-72 hours. They were then transferred to -80^{o}C storage.

A summary of the SOP is given in figure 2.1. All the above samples were processed and stored in the freezers in the TCAU to minimise delay and sample deterioration. At regular intervals these samples were logged and then moved to the university campus long term storage facility. Temperature logs were maintained for all freezers in the TCAU for internal quality control. The long term storage freezers were maintained by the university and had regular internal and external quality control checks, monitoring and alarms if out of temperature range.

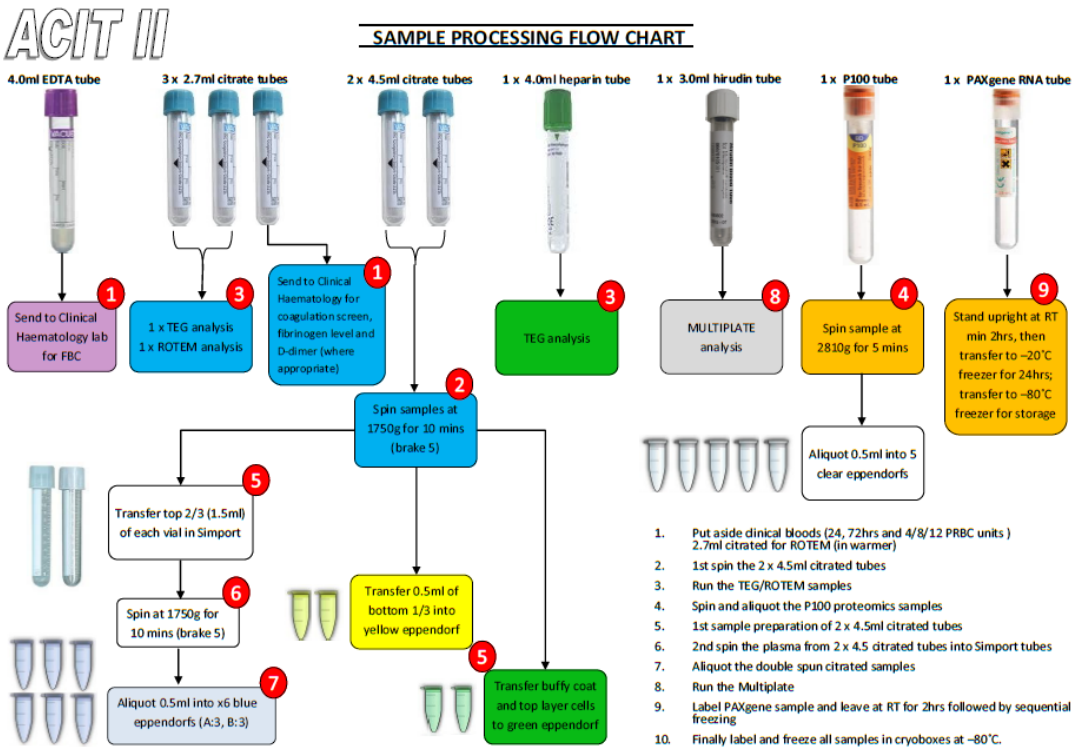


Figure 2.1 Standard operating procedure for sample processing

2.5 ROTEM analysis

Sample processing for ROTEM was carried out as per the standard automated electronic pipette program. Samples were run in all 4 available channels and data on ExTEM, InTEM, FibTEM and ApTEM were recorded. All ROTEM parameters, including those described in chapter 1 were saved for later analysis. The ROTEM Delta machines were located in the

TCAU initially and this is where samples were processed. In 2012, the ED acquired a machine in the resuscitation bay and initial samples were processed here. The citrated sample collected in a vacutainer from the patient was placed into the ROTEM delta machine which kept the blood incubated at 37^{o}C. The ROTEM reagents were removed from refrigerated storage and allowed to come up to room temperature for a period of 10 minutes prior to use: STAR-TEM (recalcitrant), ExTEM(Thromboplastin), InTEM(Ellagic acid), FibTEM(cytochalasin-D) and ApTEM(aprotinin). The machine was set up to run all 4 tests and each test well was set up with cups and pins in readiness for the test. At the appropriate time point all test wells were incubated with 300ml of blood and the STAR-TEM reagent. ExTEM, FibTEM and ApTEM were also incubated with the ExTEM reagent. The InTEM test well was incubated with InTEM reagent. The FibTEM and ApTEM then had their own reagents added. All tests were run for a period of 60 mins and all data was then recorded onto the machine. Data was downloaded every 1 – 2 weeks and stored on the university servers as a backup. The machines underwent a quality control test at regular intervals and if these showed any issues the machine use was suspended and maintenance procedures were undertaken. There were 3 machines in our TCAU (in addition to the later machine in ED). If machines had to be taken offline or if more than 1 patient sample needed to be analysed there was sufficient redundancy to continue sample processing uninterrupted. The coefficient of variation data for ROTEM is given in table 2-2. These data are provided by (GmbH, Munich, Germany) and are similar to externally validated sources(51,52)

Table 2-2 Coefficients of variation for ROTEM parameters (%)

Precision	CT	CFT	Alpha angle	CA10	MCF
Intra-assay	6	8	1	2	3
Inter-instrument	7-13	5-8	2-3	2-3	1-3

2.6 Coagulation sample assays

Where automated high volume sample processing capability existed for certain coagulation factors these were assayed by our main haematology lab using a Sysmex CS2100i analyser (Sysmex UK LTD, Milton Keynes, UK) using Siemens reagents(Siemens AG, Germany). The remainder of assays were completed manually in the form of enzyme linked immunoabsorbent assay (ELISAs). All the coagulation sample assays were conducted on the double spun (i.e. platelet depleted) plasma from the process described in section 2.4.1 of this chapter.

2.6.1 Sysmex automated system analyser

Samples assayed by our Haematology lab included PT/ International normalised ration (INR), PTT and its ratio, Fibrinogen (Fg), Factor II, Factor V, Factor VII, Factor VIII, Factor IX, Factor X, Factor XI, Factor XIII, Von Willebrand factor Antigen (VWF:Ag), Antithrombin (AT), Protein C (PC), Free Protein S Antigen (PS), Soluble Fibrin Monomer Complexes (SFMCs), A2AP and D-Dimer. The specific methods, reference ranges and variability is summarised in table 2-3. It is important to note that the tests for the numbered coagulation factors ie; II, V, VII, IX, X, XI, and XIII measure activity rather than concentration. The sample processing was carried out in 2 main batches. The first batch of samples was from the RLH only. This batch includes up to patient number 324 in our recruitment database. Samples were subsequently processed in a second much bigger batch to include all other samples. The 2 batches had marginally different references ranges and both of these are given here. It is notable, however, that the variability is still the same and in fact the means in the references ranges are identical in many cases. In the remainder the variation is negligible.

Table 2-3 Summary of all sample assays carried out by Sysmex CS2100i automated analyser

Parameter	Reference Range RLH 0-324	Reference Range After RLH 325 & All INTRN samples	Units	Reagent	Reagent Supplier	Method	Intra-assay variability (normal sample)	Inter-assay variability (normal sample)
PT	9.4-12.4	9.4-12.2	secs	Siemens Innovin	Sysmex UK	Clotting	1.3%	**2.0%**
INR	0.9-1.1	0.9-1.1	ratio					
APTT	21-31	21-31	secs	Siemens Actin FS	Sysmex UK	Clotting	4%	**4%**
APTT ratio	0.8-1.2	0.8-1.2	Ratio					
Fibrinogen	1.50-4.50	1.56 – 4.0	g/l	Siemens Thrombin	Sysmex UK	Clauss	5.9%	**2.3%**
Factor II	78-117	70 – 146	iu/dL	Siemens Innovin and Siemens Factor II deficient plasma	Sysmex UK	Clotting by multi-dilution analysis	2%	**7%**
Factor V	66-114	62 – 150	iu/dL	Siemens Innovin and Siemens Factor V deficient plasma	Sysmex UK	Clotting by multi-dilution analysis	2%	**7%**
Factor VII	50-150	67 – 143	iu/dL	Siemens Innovin and Siemens Factor VII deficient plasma	Sysmex UK	Clotting by multi-dilution analysis	2%	**7%**
Factor VIII	52-153	52 – 153	iu/dL	Siemens Actin FS and Siemens Factor VIII deficient plasma	Sysmex UK	Clotting by multi-dilution analysis	2%	**7%**
Factor IX	58-138	58 – 138	iu/dL	Siemens Actin FS and Siemens Factor IX deficient plasma	Sysmex UK	Clotting by multi-dilution analysis	2%	**7%**
Factor X	50-150	65 – 152	iu/dL	Siemens Innovin and Siemens Factor X deficient plasma	Sysmex UK	Clotting by multi-dilution analysis	2%	**7%**
Factor XI	58-148	58 – 148	iu/dL	Siemens Actin FS and Siemens Factor XI deficient plasma	Sysmex UK	Clotting by multi-dilution analysis	2%	**7%**
Factor XIII	70-14	55 – 155	iu/dL	Siemens Berichrom F XIII	Sysmex UK	Chromogenic method	5%	**6%**
Von Willebrand Antigen	50-160	50 – 156	iu/dL	Siemens vWF Ag	Sysmex UK	Latex immunoassay	1.4%	**0.9%**
Antithrombin	81-119	81 – 119	iu/dL	Siemens Berichrom Antithrombin III (A)	Sysmex UK	Chromogenic	1.3%	**4.6%**
Protein C	72-162	72 – 162	iu/dL	Siemens Berichrom Protein C	Sysmex UK	Chromogenic	1.3%	**1.9%**
Free Protein S Antigen	62-120	60 – 139	iu/dL	Stago Liatest Free Protein S	Diagnostica Stago	Latex immunoassay	2.4%	**2.6%**
α_2-antiplasmin	76-126	68 – 136	iu/dL	Siemens Berichrom α_2-antiplasmin	Sysmex UK	Chromogenic	0.5%	**3.2%**
Soluble Fibrin Monomer Complexes	<6	<6	µg/mL	Stago Liatest Fibrin Monomer	Diagnostica Stago	Latex immunoassay	2.5%	**3.6%**
D-Dimer	<550	0 – 440	ng/mL	Siemens Innovance D-Dimer	Sysmex UK	Latex immunoassay	4.1%	4.3%

2.6.2 Coagulation sample ELISAs

Samples assayed in our own lab as ELISAs included tPA, Prothrombin fragments 1+2 (PF1+2), Thrombomodulin (TM), Plasmin anti plasmin complex (PAP), Plasminogen activator inhibitor-1 (PAI-1), Thrombin activatable Fibrinolysis inhibitor (TAFI) and activated Thrombin activatable Fibrinolysis inhibitor (TAFIa). All ELISAs were carried out by 4 trained staff including myself under the supervision of our laboratory manager. Assays were completed as per the instructions for each test with no deviation. Where tests were above or below detection thresholds and we had further sample available, repeat assays were carried out with sample diluted further to allow detection within the range of the ELISAs. A summary of the ELISAs used is given in table 2-4 below. Of note the Thrombomodulin ELISA was first run using the commercially available kit provided by Diagnostica Stago (Asniers-Surs-Seine, France). However on the second batch of testing the Stago ELISA kit was no longer in production and an alternative had to be sought. The second batch of tests (beyond RLH pt number 324) was run using the ELISA kit from Abcam (Cambridge, UK). Both ELISAs are shown in the table separately.

Table 2-4 ELISA characteristics for all assays

Parameter	Reference Range RLH 0-324	Reference Range RLH 325+ & All INTRN samples	Units	Reagent	Reagent Supplier	Method	Intra-assay variability (normal sample)	Inter-assay variability (normal sample)
TAFI	7.6-10.6	Not done	µg/mL	Asserachrom TAFI	Diagnostica Stago	Immunosorbent assay	4.3%	6.9%
TAFIa	8.53-22.07	Not done	ng/mL	Asserachrom TAFIa	Diagnostica Stago	Immunosorbent assay	3.4%	3.15%
PAP	120-700	120-700	µg/L	PAP micro	DRG instruments, GmbH, Germany	Immunosorbent assay	4.2%	7.25%
tPA	2–12	2–12	ng/mL	Asserachrom tPA,	Diagnostica Stago	Immunosorbent assay	4.515%	6.395
PF 1+2	69-229	69-229	pmol/L	PF 1+2; Enzygnost F1+2 monoclonal	Siemens Healthcare Diagnostics	Immunosorbent assay	3.6-5.5%	4.4-11.2%
TM up to RLH 324	27-59	-	ng/ml	Asserachrom Thrombomodulin	Diagnostica Stago	Immunosorbent assay	2.5-5.1%	7.9-10.2%
TM RLH325 onwards & all INTRN site samples	-	2.9 – 7.6	ng/ml	Thrombomodulin	Abcam	Immunosorbent assay	3.9%	9.8%
PAI-1	4-43	4-43	ng/ml	Asserachrom PAI-1	Diagnostica Stago	Immunosorbent assay	5.48-6.53%	6.52-8.69%

All ELISAs were carried out in the Queen Mary University Blizzard Labs. Common equipment in all tests were the multipipette used manually and an auto strip ELx50 plate washer (BioTek, Winooski, USA).

2.6.3 Data Acquisition

All ELISA data were collected as absorbance in duplicate at the suggested light spectrum length on a SIAFR Synergy HT plate reader (BioTek, Winooski, USA) using KC4 v3.4 software, plotted against the dose and a standard curve fitted by linear regression analysis using GraphPad Prism program version 4.0 (GraphPad Software Inc., San Diego California USA) in accordance with the manufacturers' and GraphPad Prism Instruction Manual. Mean absorbance was converted to the relevant units (e.g. pmol/L) of the given coagulation protein using linear regression.

2.7 Data Collection

2.7.1 Patient demographics and injury characteristics

At the time of blood sample collection and recruitment, heart rate, systolic blood pressure (SBP), respiratory rate, Glasgow coma score (GCS), temperature, time of injury and time of blood draw were recorded. Full patient demographics, mechanisms of injury, injury type and severity were collected from the RLH trauma registry as soon as available. Injuries were scored and classified according to AIS to work out a composite ISS(53) .

2.7.2 Fluid therapy in the first 24 hours

All intravenous (IV) fluid therapy as well as blood or blood product transfusions were recorded for the first 24 hours of admission. These were broken down into crystalloids, colloids, hypertonic saline (HTS), packed red blood cells (PRBCs), FFP, Cryoprecipitate (Cryo) or platelets.

2.7.3 Outcomes measures

Outcome measures were collected including 28 day outcome (discharge or death), total LOS, critical care days, ventilator free days, vasopressor days and renal replacement therapy (RRT) days

2.8 Statistical analysis

All analysis was carried out using GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla CA, US), Microsoft Excel 2010 (Microsoft Inc., Redmond WA, US) and MATLAB (Mathworks, Natick, MA, USA). Parametric data are expressed as mean ± standard deviation (SD) or 95% confidence intervals. Non parametric data are expressed as median and interquartile range. For comparison between two groups analysis was performed using two-tailed unequal variance student's t test for and Mann-Whitney U for parametric data non-parametric data respectively. For more than two group comparison the Kruskal-Wallis test was used and no assumption was made about the normality of the groups. For analysis of contingency data, a x^2 test for trend was used. A P-Value of <0.05 was used to represent statistical significance throughout this work.

Chapter 3

The incidence and magnitude of fibrinolytic activation in trauma patients

3.1 Introduction

Trauma is a global public health problem leading to millions of deaths a year. Forty per cent of trauma deaths are due to bleeding and occur in the first few hours after injury(18). Haemostatic dysfunction is common and up to 25% of severely injured trauma patients have an established coagulopathy when they arrive in the ED(24). This early coagulopathy is independently associated with mortality, increased transfusion requirements, organ injury, septic complications and critical care stays(25). Understanding the aetiology of acute traumatic coagulopathy and all aspects of haemostatic dysfunction associated with trauma is a current imperative in the management of these patients.

Fibrinolysis is known to occur during traumatic coagulopathy but the true incidence and magnitude of fibrinolytic activity (FA) in trauma has never been studied in detail(30). Screening laboratory tests of coagulation (e.g. PT, PTT) do not assess fibrinolysis and near patient global assays such as thromboelastometry are not in widespread routine use. The few small studies that have investigated the use of rotational thromboelastometry (ROTEM) in trauma suggest that the measure of FA used, known as ROTEM hyperfibrinolysis, is rare with an admission incidence of 3-6%(43,49,50). However abnormal laboratory coagulation results are common in trauma patients and we have previously shown their association with an increased fibrinolytic potential(30). This suggests the underlying incidence and magnitude of fibrinolysis may be more common than considered previously.

Hyperfibrinolysis is classically defined as when fibrinolytic activity is potentially greater that fibrin formation, such that clot integrity is threatened. Hyperfibrinolysis is identified on ROTEM when maximum lysis (ML) exceeds 15% of the maximum clot firmness(51). However there has never been an attempt to correlate this definition with classic laboratory tests of fibrinolysis. Trauma patients with ROTEM hyperfibrinolysis have mortality rates of 80-100%, even when treated(49). However, clinical trials of

antifibrinolytic therapy suggest that activation of fibrinolysis is more prevalent. Trauma patients randomised to tranexamic acid in the CRASH-2 trial had a 9% improved survival compared to those who received placebo(34). Despite these findings some trauma units only administer antifibrinolytics if 'ROTEM hyperfibrinolysis' is present. On the basis of the results from CRASH-2, we hypothesized that trauma patients have increased FA that is undetectable by ROTEM which might explain the benefit from tranexamic acid administration.

The objectives of the current study were to determine the prevalence and severity of FA in trauma as measured by increases in plasmin generation, as measured by plasmin-antiplasmin levels (PAP). Secondly we wished to determine the ability of the ROTEM to detect FA. Lastly we wished to assess whether FA was associated with the severity of injuries, admission physiology and clinical outcomes.

3.2 Methods

We conducted a prospective observational cohort study of trauma patients presenting to a single major trauma centre. The study was reviewed and approved by the UK Regional Ethics Committee. Between January 2007 and June 2009 all adult trauma patients (>15 years) who met the local criteria for trauma team activation were eligible for enrolment into the study. Exclusion criteria were arrival in the ED >2 hours following injury; >2000ml intravenous fluid prehospital; transfer from another hospital and burns >5% total body surface area. Patients were retrospectively excluded if they declined to give consent to use research samples; were receiving anticoagulant medications (not including aspirin); had moderate or severe liver disease or a known bleeding diathesis.

We categorized patients according to the degree of increased fibrinolytic activity based on the level of PAP complexes and ROTEM hyperfibrinolysis. Expected normal values for PAP levels as given by the manufacturer were 120–700μg/L (2.5%-97.5% percentiles). PAP

thresholds were arbitrarily set at approximately twice the upper limit of normal for moderate FA (>1500µg/L), in order to exclude minor elevations that might be expected in these patients. ROTEM hyperfibrinolysis was defined as ML >15%(51).

Patterns of FA were classified as;

- 'Normal' (PAP ≤ 1500 µg/L & ML <15%)
- 'Moderate' with FA only (PAP >1500 µg/L & ML <15%)
- 'Severe' with FA and ROTEM lysis (PAP >1500 µg/L & ML >15%)
- ROTEM only lysis (PAP ≤ 1500 µg/L & ML >15%)

A 20mL research sample of blood was drawn from either the femoral vein or antecubital fossa along with the standard trauma laboratory tests within 20 minutes of arrival in the ED. Blood for thromboelastometry analysis was drawn into a 2·7ml citrated vacutainer (0·109M buffered sodium citrate, 3·2% - Becton Dickinson, Plymouth, UK). Samples for PT were collected into 4·5ml glass vacutainers (0·109M buffered sodium citrate, 3·2% - Becton Dickinson, Plymouth, UK), volume 9:1. The PT was processed on the Sysmex CS2100i automated analyser. The sample for haemostatic assays was placed in a citrated tube and spun down within two hours of blood draw. First at 1750g for 10 minutes, the supernatant was then extracted and respun at 1750g for a further 10 minutes. The extracted plasma was stored in aliquots at -80°C freezer. Blood gas analysis for Base Deficit (BD) was performed simultaneously with the research sample collection.

ROTEM samples were processed within one hour of blood draw at 37°C on a ROTEM delta instrument (TEM International GmbH, Munich, Germany) and all treating clinicians were blinded to the results. The methodology and the parameters of ROTEM have previously been described in detail(54) and in chapter 2.5 above. Two separate ROTEM assays were performed for each patient, the EXTEM measuring tissue factor initiated clotting and the APTEM with the addition of an antifibrinolytic. For the EXTEM, 20µl of 0.1M $CaCl_2$

(STARTEM) and 20µl of tissue factor derived from rabbit brain were placed into the test cuvette after which 300µl of the blood sample was added. The APTEM test analysis was performed in the presence of 20µl aprotinin. Mild activation with tissue factor is performed to standardize the in vitro coagulation process and produce a more rapid result. All pipetting steps and the mixing of reagents with samples were guided by the electronic pipette program. Clot breakdown was determined by rate of maximum lysis (ML) during 60 minute assay time for each sample analysed. Plasma activity levels of α2-antiplasmin (Siemens Berichrom α_2-antiplasmin, Sysmex, UK) were assayed using a Sysmex CS2100i automated analyser. Latex immunoassays were used to quantify the levels of D-Dimer (Siemens Innovance D-Dimer, Sysmex, UK) also using Sysmex CS2100i automated analyser. Enzyme linked immunosorbant assays were used to quantify tissue plasminogen activator (tPA ; Asserachrom tPA, Diagnostica Stago, France); plasmingen activator inhibitor- 1(PAI-1; Asserachrom PAI-1, Diagnostica Stago, France); prothrombin fragments 1+2 (PF 1+2; Enzygnost F1+2 monoclonal, Siemens Healthcare Diagnostics, Germany); plasmin-α2-antiplasmin complex (PAP; DRG PAP micro, Germany); thrombin activatable fibrinolysis inhibitor(TAFI; Asserachrom TAFI, Diagnostica Stago, France); and activated thrombin activatable fibrinolysis inhibitor(TAFIa; Asserachrom TAFIa, Diagnostica Stago, France)

Data were collected prospectively on patient demographics, time of injury, mechanism (blunt or penetrating), prehospital fluid administration, time of arrival in the ED and baseline vital signs. Injury was classified using the ISS(53) as moderate (<16) severe (16-24) or critical (>24) trauma. Arterial or venous blood gas base deficit was used a marker of tissue hypoperfusion. Patients were followed until hospital discharge or death. Outcome measures recorded were 28-day mortality, 28-day ventilator-free days and all transfusions required in the first 24 hours.

Normal-quantile plots were used to test for normal distribution in patients with mild injuries and no shock. Parametric data are expressed as mean (confidence intervals). For the parametric data two-group analysis was performed with a two tailed unequal variance Student's *t* test. Injury severity and its components (AIS), ventilator free days and hospital length of stay were not normally distributed and expressed as median (interquartile range). For the non-parametric data, 1 way ANOVA (Kruskal-Wallis) was used for analysis. Univariate and multiple regression analyses were used to assess correlation. When analysing contingency data, χ^2 test for trend was used. A *p* value of <0·05 was chosen to represent statistical significance throughout.

3.3 Results

Three hundred and twenty three trauma patients were eligible for enrolment into the study over an 18-month period. 4 subjects declined consent, 3 were discharged early with no contact details, 6 were unable to give consent and had no next of kin, 5 were unable to consent and their next of kin declined on their behalf and a further 2 patients were excluded due to protocol violation. Of the 303 remaining patients, 10 did not have ROTEM studies performed, 5 were unable to have ROTEM studies leaving 288 patients included in the study. Patient demographics and injury characteristics are shown in Table 3-1. The admission physiology and outcomes are summarised in Table 3-2. The rate of blunt trauma and mortality increased with the magnitude of lysis, all of those with severe lysis had suffered blunt trauma and carried a mortality of 40%. The admission temperature in all patients was slightly below the normal range but did not vary significantly between the groups.

Table 3-1 Patient Demographics and Injury Characteristics

Values are number (%), mean (confidence intervals) or percentage of patient group. ISS and AIS are given as median (interquartile range). ISS: Injury Severity Score, AIS: Abbreviated Injury Score. '*' denotes p <0.05 vs Normal, '+' denotes p<0.05 moderate vs severe lysis.

	ALL PATIENTS	NORMAL	MODERATE	SEVERE	ROTEM LYSIS ONLY
Number	303	100 (35%)	165 (57%)	15 (5.2%)	8 (2.7%)
Age	37 (36-39)	32 (30-35)	40 (37-43)*	40 (33-47)	44(36-53)
% Male	81.9	85.0	80.0	73.3	87.5
Injuries					
% Blunt Trauma	79.5	63.0	88.5*	93.3*	50
ISS	10 (4-25)	6 (1-10)	17 (9-28)*	25 (17-38)*	5 (2-9)
% ISS >15	41.9	12.2	55.8*	86.7*	0
AIS Extremity	1 (0-3)	0 (0-2)	2 (0-3)*	3 (1-3)*	1 (0-2)
AIS Thorax	0 (0-3)	0 (0-1)	2 (0-3)*	3 (0-5)*	0 (0-0)
AIS Head	0 (0-2)	0 (0-1)	0 (0-3)*	0 (0-3)	0 (0-0)

Table 3-2 Admission physiology and outcomes

Values are mean (confidence intervals) or percentage of patient group. Ventilator free days and hospital stay are given as median (interquartile range). SBP: Systolic Blood Pressure, PT: Prothrombin Time, ROTEM CT: Clotting time on ROTEM, ROTEM CA5: clot amplitude at 5 minutes on ROTEM, PRBC: Packed Red Blood Cells, FFP: Fresh Frozen Plasma. '*' denotes p <0.05 vs Normal, '+' denotes p<0.05 moderate vs severe lysis.

	ALL PATIENTS	NORMAL	MODERATE	SEVERE	ROTEM LYSIS ONLY
Admission physiology					
Temperature(°C)	35.8(35.6-36.0)	36.0(35.8-36.2)	35.7(35.3-36.0)	35.1(34.0-36.3)	36.0(35.3-36.6)
SBP (mmHg)	130 (127-133)	130 (125-136)	130 (125-136)	119 (100-138)	141 (128-155)
Base Deficit (mmol/l)	2.4 (1.8-2.9)	0.5 (-0.1–1.1)	2.9 (2.1-3.7)*	8.6 (5.2-12.0)*+	0 (-2.3-2.6)
PT (seconds)	11.6 (11.5-11.8)	11.2 (11.1-11.4)	11.7 (11.5-12.0)*	13.6 (11.8-15.4)*	11.3 (10.8-11.7)
% PT ratio >1.2	9.4	3.0	10.9*	40.0*	0.0
ROTEM CT	70.0(64.8-75.1)	72.0(65.5-78.6)	67.4(59.6-75.1)	84.7(61.5-108.0)	69.9(44.0-95.7)
ROTEM CA5	42 (41-43)	43 (41-45)	42 (41-43)	35 (30-41)*+	41 (29-51)
24-Hour transfusions					
PRBC (units)	1.7 (0.6-2.7)	0.2 (0.0-0.7)	2.0 (1.3-2.7)*	6.5 (3.0-10.0)*+	0 (0-0)
FFP (units)	0.8 (0.1-1.5)	0.1 (0.0-0.9)	1.0 (0.6-1.4)*	2.9 (1.2-4.5)*	0 (0-0)
Platelets (units)	0.2 (0.0-0.4)	0.0 (0.0-0.0)	0.2 (0.1-0.3)*	0.7 (0.3-1.0)*+	0 (0-0)
Cryoprecipitate (units)	0.2 (0.0-0.4)	0.0 (0.0-0.0)	0.2 (0.1-0.4)*	0.6 (0.2-1.0)*	0 (0-0)
Outcomes					
28-day Mortality %	8.9	1.0	12.1*	40.0*	0.0
28-day Ventilator-free days	28 (27-28)	28(28-28)	28 (27-28)*	28 (27-28)	28 (27-28)
Hospital stay (survivors)	15 (12-18)	2 (1-6)	11 (4-25)*	26 (15-32)*	4 (1-9)

3.3.1 Rates of Fibrinolytic Activation and ROTEM lysis

Fifteen of the patients (5%) had ROTEM hyperfibrinolysis, and of these four patients (1%) had a ML >50% (Figure 3.1A). 180 patients (59%) had evidence of FA in that they had PAP levels over 1500 μg/L. 165 patients (57%) had increased PAP levels (>1500μg/L) without ROTEM hyperfibrinolysis (Figure 3.1B). Significant increases in ML occurred when PAP levels were above 20,000 μg/L (Figure 3.1C). When aprotinin was added *in vitro* to the plasma sample, there was an improvement in ML only in this group. Active fibrinolysis was evidenced in the moderate group by markedly increased D-Dimer levels (Table 3-3). Plasmin generation was associated with significantly increased fibrin breakdown as measured by D-Dimer production regardless of whether lysis was visible on thromboelastometry (Figure 3.1D). There was a ROTEM-only lysis group with 8 patients (2.7%) that showed no FA. They had low mean PAP levels (1011 μg/L) and no evidence of fibrin breakdown as characterised by D-Dimer (mean 865ng/mL). In fact this was lower than the normal group.

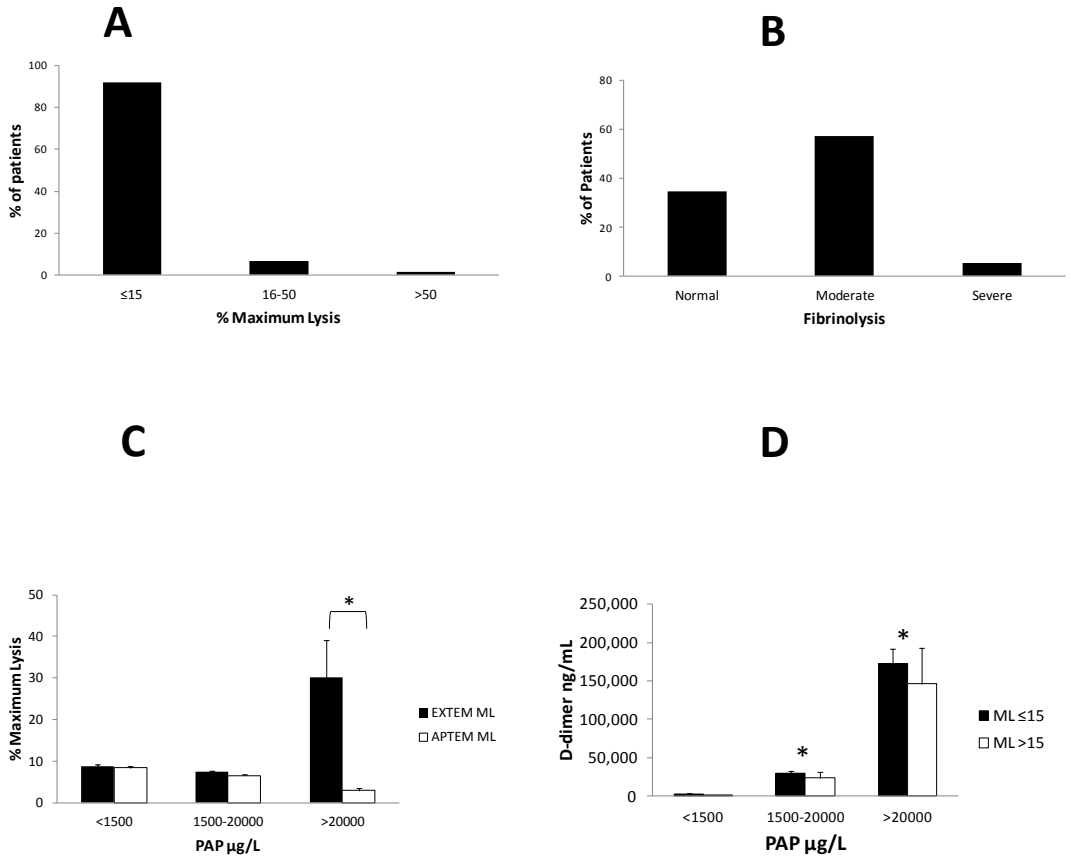


Figure 3.1 Thromboelastometry underestimates the incidence and severity of fibrinolysis

A: Incidence of hyperfibrinolysis as defined by functional lysis on ROTEM (ML)

B: Incidence of moderate and severe hyperfibrinolysis (Normal: ROTEM ML<15% and PAP<1500µg/L; Moderate: ML<15% and PAP>1500µg/L; Severe: ML>15% and PAP>1500µg/L)

C: Functional clot lysis (dark bars) was only measureable with very high PAP levels (ML%: PAP<1500µg/L - 8.8% vs PAP>20,000µg/L - 30.3%). Functional clot lysis improvement with aprotinin (white bars) was only identified at high plasmin levels (%ML: PAP<1500 - 0.5% vs PAP >20,000µg/L - 27.3%; *p<0·001 vs PAP<1500µg/L).

D: D-Dimer levels were equivalent whether or not lysis was detected on thromboelastometry for given plasmin generation levels (D-Dimer: PAP 1500-20000 - ML≤15% 29,167 vs ML>15% 23,390 p=0.85; PAP>20000 –ML≤15% 171,971 vs ML>15% 146,093 p=0.62; *p<0.05 compared to PAP<1500µg/L). Values are % or mean + 95% confidence interval.

Table 3-3 TEM and Coagulation factor assays

Values are mean (confidence intervals). ML: maximum lysis. PAP: plasmin-antiplasmin complex (normal range 120-700 µg/L). tPA : tissue plasminogen activator (normal range 2–12 ng/mL). D-Dimer (normal range <550ng/mL). Antiplasmin (α2) (normal range 76-126 iu/dL). PAI-1: Plasminogen activator inhibitor-1 (normal range 4-43 ng/mL). PF1+2: prothrombin fragments 1+2(normal range 69-229 pmol/L). TAFI: thrombin activatable fibrinolysis inhibitor (normal range 7.6-10.6 µg/mL). TAFIa: activated thrombin activatable fibrinolysis inhibitor (normal range 8.53-22.07 ng/ml). '*' p <0.05 vs Normal, '+' denotes p<0.05 moderate vs severe lysis.

	ALL PATIENTS	NORMAL	MODERATE	SEVERE	ROTEM LYSIS ONLY
ML	9.4 (8.0-10.8)	8.1 (7.4-8.7)	6.6 (6.0-7.1)*	45.5 (28.0-63.0)*+	18.4 (16.5-20.3)*
PAP	4690 (3887-5494)	928 (862-993)	5844 (4835-6854)*	17503 (10502-24503)*+	1011 (794-1227)
tPA	11.8 (10.2-13.5)	8.0 (6.8-9.1)	12.4 (10.8-14.0)*	39.1 (14.5-63.7)*	8.3 (3.8-12.7)
D-Dimer	30544 (22822-38266)	2576 (1727-3426)	38687 (30502-46872)*	88831 (31348-146315)*	865 (488-1243)*
Antiplasmin	122.8 (119.6-126.1)	131.4 (127.6-135.2)	120.4 (116.0-125.0)*	86.7 (66.0-107.3)*+	130.4 (119.9-141.0)
PAI-1	32.7(28.0-37.4)	38.3(27.7-48.9)	29.6(24.6-34.6)	28.6(21.4-35.8)	43.7(0.1-87.4)
PF1+2	1865 (1562-2168)	596 (489-703)	2314 (1911-2716)*	5062 (2546-7577)*	316 (234-399)*
Fibrinogen	2.1 (2.0-2.1)	2.2 (2.1-2.3)	2.1 (1.9-2.1)*	1.5 (1.1-1.9)*+	2.1 (1.7-2.4)
TAFI	12.6 (12.2-13.0)	13.5 (12.9-14.1)	12.0 (11.5-12.5)*	10.6 (9.0-12.4)*	13.3 (11.1-15.3)
TAFIa	132 (117-147)	84 (66-103)	153 (133-173)*	303 (179-427)*+	66.9 (58.2-75.5)

3.3.2 Relationship to injury characteristics

Increasing injury severity score was associated with increasing FA, as measured by both PAP levels and ML (Figure 3.2A). However ROTEM ML increased only minimally, from 6% with ISS≤15 to 12% for ISS>24 (p=0·10). In contrast PAP levels rose just over 5-fold from 2036µg/L (ISS≤15) to 10697µg/L (ISS>24, p<0·001). 90% of patients with an ISS>24 had PAP levels >1500µg/L, but in only 11·6%had ROTEM hyperfibrinolysis (Figure 3.2A), showing that PAP was more sensitive to injury severity than ROTEM. In a multiple regression of all organ injuries, PAP levels correlated with the severity of head, chest and extremity trauma (p<0·001 all 3 regions; r^2=0·28): whereas %ML was very weakly correlated with the severity of extremity injury only (p=0·008, r^2=0·05). PAP levels correlated with the degree of shock as well as injury severity (BD p<0·001; ISS p<0·001; r^2=0·36, Figure 3.2B). 88% of patients with a BD >6mmol/l had moderate fibrinolysis compared to 58% of patients with BD <6mmol/l (p<0.0001, Figure 3.2C). D-Dimer levels were high even with relatively low levels of shock (Figure 3.2D). ROTEM maximum lysis levels were not correlated with the degree of shock as measured by the base deficit or systolic blood pressure. Low SBP was also associated with increasing FA (Figure 3.2E) and 91% of patients with a SBP <90mmHg had moderate or worse FA compared to 59% with SBP >90mmHg (p =<0.0001, Figure 3.2F). Mild FA occurred at moderate levels of both injury severity and shock, whereas severe hyperfibrinolysis only occurred in association with the most severe extremity injuries. ROTEM-only lysis followed the same injury pattern as no lysis with low levels of shock and low injury severity.

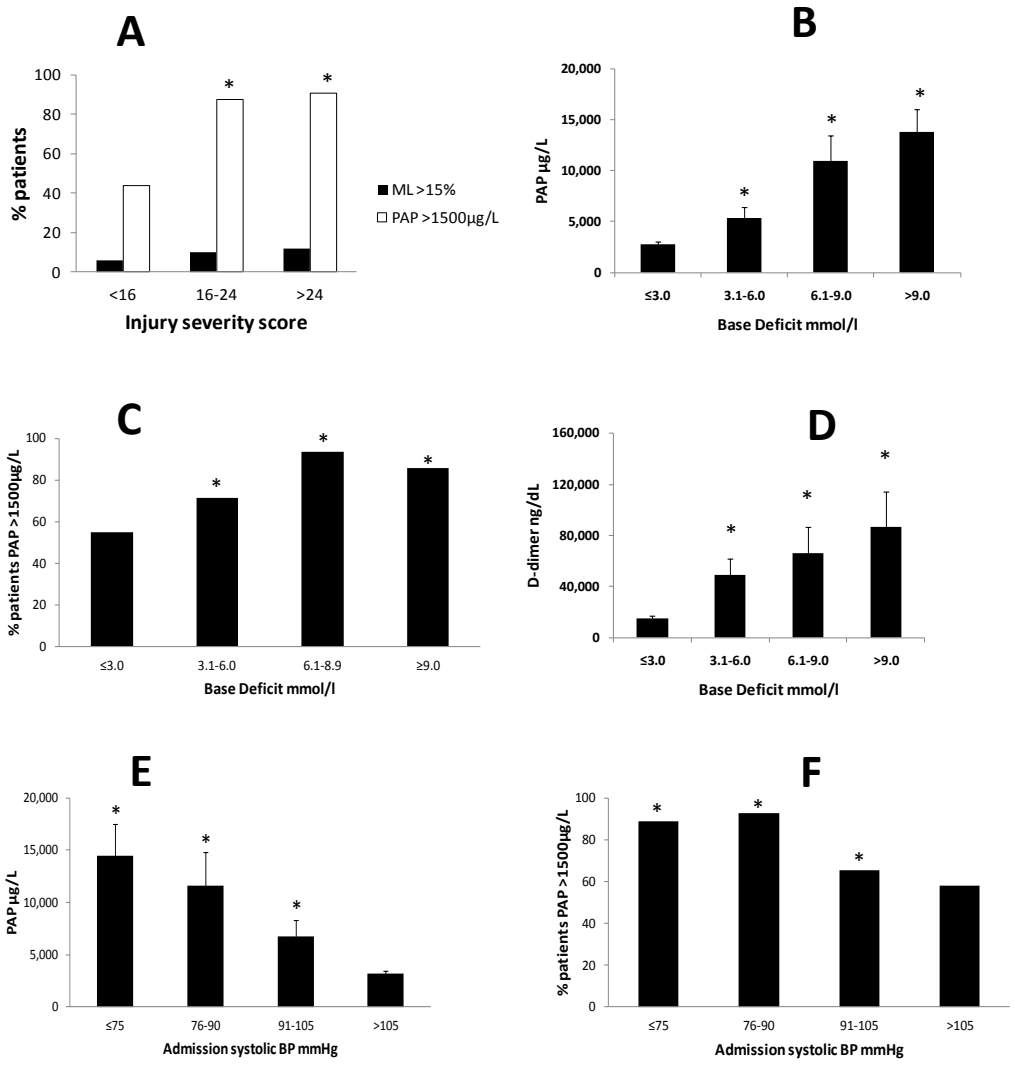


Figure 3.2 Fibrinolytic activation is associated with injury severity and the degree of shock

A: Proportion of patients presenting with high ML and PAP levels by injury severity scores. %patients with overt fibrinolysis (ML>15%) rose slightly with injury severity (ISS <16: 5.8%, ISS 16-24: 10.0%, ISS>24: 11.6%, p >0.05), while %patients with PAP>1500µg/L rose substantially with injury severity (ISS <16: 43.8%, ISS 16-24: 87.5%, ISS>24: 90.9%, p <0.001)

B: PAP levels increased with the degree of systemic hypoperfusion (*p <0.001 vs BD <3.0mmol/l).

C: Proportion of patients presenting with high PAP (>1500µg/L) increased with systemic hypoperfusion (BD<3.0: 55%, BD 3.1-6.0: 71%, BD 6.1-9.0: 93%, BD>9.0: 86%, P<0.001 vs BD <3.0mmol/l)

D: D-Dimer levels increased significantly with increasing hypoperfusion. Even at moderate hypoperfusion (BD 3.1-6.0: D-Dimer 49,387ng/mL) levels were very high and increased substantially as hypoperfusion increased (BD 6.1-9.0: D-Dimer 66,233ng/mL, BD>9.0: D-Dimer 86,892ng/mL p<0.001 vs BD <3.0mmol/l)

E: PAP levels were inversely related to SBP. Even at borderline SBP, PAP levels were significantly higher and continue to rise as SBP falls (SBP >105mmHg: 3170µg/l; SBP 91-105mmHg: 6776µg/l; SBP 76-90mmHg: 11029µg/l; SBP ≤75: 14,876 µg/l, p<0.001 vs SBP >105mmHg)

F: Proportion of patients with high PAP (>1500µg/L) increases with falling blood pressure (SBP >105mmHg: 58%; SBP 91-105mmHg: 66%, SBP 76-90mmHg: 92%, SBP ≤75mmHg: 89%; p<0.0001 vs SBP >105). Values are % or mean + 95% confidence interval.

3.3.3 Mechanisms of activation

Both tPA and PAP levels were significantly incrementally elevated as expected in all those with FA (Table 3-3). Levels of PF1+2 (Table 3-3) also incremented in the same pattern as FA, suggesting that thrombin might be a key stimulator of tPA release from the endothelium. Levels of tPA correlated with hypoperfusion as well as injury severity (ISS: p=0·002; BD: p<0·001; r2=0·19). Severe FA was associated with massive tPA production and PAP levels such that there was significant consumption of antiplasmin (Figure 3.3A). Severe hyperfibrinolysis was only seen in association with α2-antiplasmin activity below 75iu/dL (Figure 3.3B). However significant D-Dimer generation continued to occur whatever the level of α2-antiplasmin activity (up to 125iu/dL , Figure 3.3C). TAFI levels did not seem to strongly affect the regulation of fibrinolysis as D-Dimer levels were negatively correlated with TAFI levels and positively correlated with TAFIa levels (Figure 3.3D). ROTEM only lysis again followed the pattern of normal lysis tPA and PAP not raised and α2-antiplasmin not reduced.

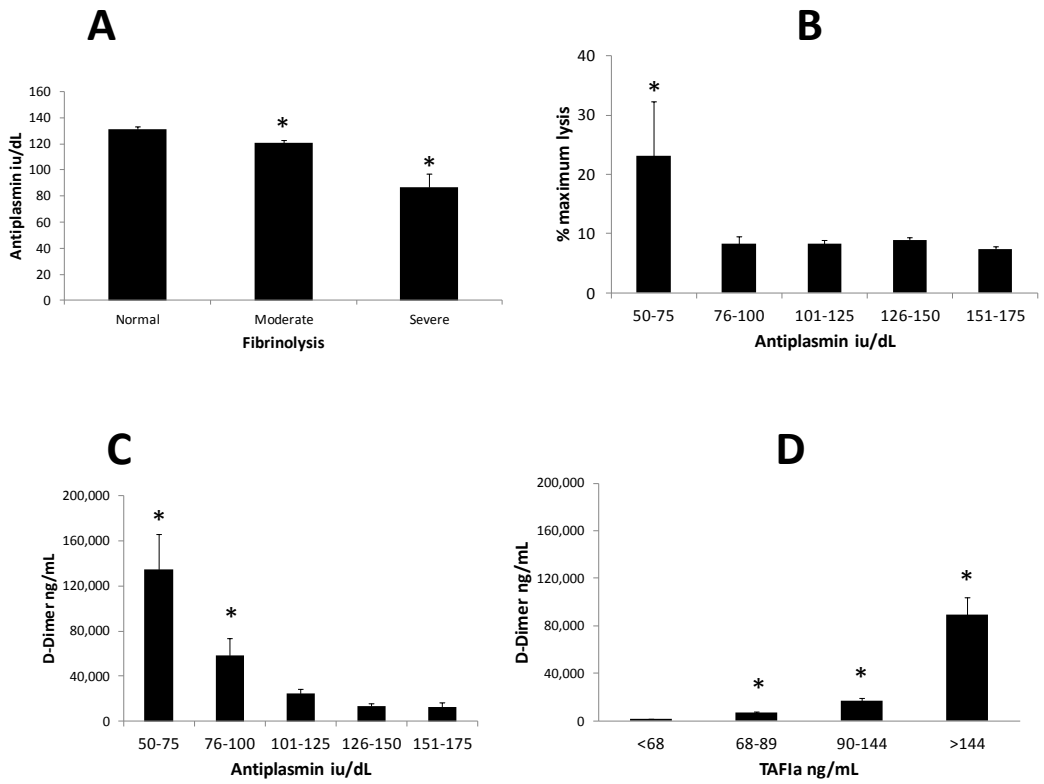


Figure 3.3 Mechanisms of activation of moderate and severe fibrinolysis

A: Antiplasmin activity fell in Moderate fibrinolysis but was only 66% of normal in the Severe group (Normal 131iu/dL; Moderate 120iu/dL; Severe 87iu/dL, *p<0.001 vs Normal).

B: ROTEM hyperfibrinolysis was only seen when antiplasmin activity fell below 75iu/dL (%ML: AP 50-75iu/dL : 23.1%, AP: 151-175iu/dL: 7.4%, *p<0.01 vs AP 151-175iu/dL).

C: In contrast, D-Dimer levels were raised at relatively normal activity of antiplasmin (D-Dimer - AP 50-75iu/dL: 135,161ng/mL; AP 76-100iu/dL: 58,606ng/mL; AP 101-125iu/dL: 24,713ng/mL; *p<0.05 vs AP 150-175iu/dL: 12,547ng/mL).

D: TAFIa levels were positively correlated with D-Dimer production (TAFIa in quartiles, *p<0.001 vs 1st quartile).

3.3.4 Clinical outcomes

The small number of patients with severe FA had a high mortality (6/15, 40%), but those with moderate FA were a larger group and despite having a lower rate of deaths, had a higher number of deaths overall (20/165, 12%). Patients with moderate FA had a 12-fold increase in 28-day mortality compared to patients with no lysis (Figure 3.4, Table 3-2). PAP levels were independently associated with 28-day mortality in a multiple regression analysis including age, mechanism of injury, injury severity, admission systolic blood pressure and base deficit (r2=0·24; PAP p<0·001). Of the patients who died in the severe FA group the average time to death was less than a day and all deaths occurred within 48 hours.

There was an association between severity of fibrinolysis and the average number of blood transfusions per patient (Figure 3.4B). PAP levels were independently associated with 24-hour red cell requirements in a multiple regression analysis including mechanism of injury, injury severity, admission systolic blood pressure, base deficit and prothrombin ratio (r2=0·24; PAP p<0·001). Patients with moderate FA were also more likely to receive plasma (20% vs 2%, p<0·001 - Figure 3.4C), platelet (12.7% vs 0%, p<0·001) and cryoprecipitate transfusions (11.5% vs 0%, p<0·001). Compared with the 'normal' group, survivors with moderate FA had increased incidence of septic complications (48% vs 3%, p<0·001), fewer 28-day ventilator-free days (27 vs 28 days, p<0·028) and spent longer in hospital (11 vs 2 days, p<0·001 - Figure 3.4D). The ROTEM only lysis group again reflected a relatively uninjured cohort with 0% mortality, no use of blood or products and a median length of stay just over 48hrs.

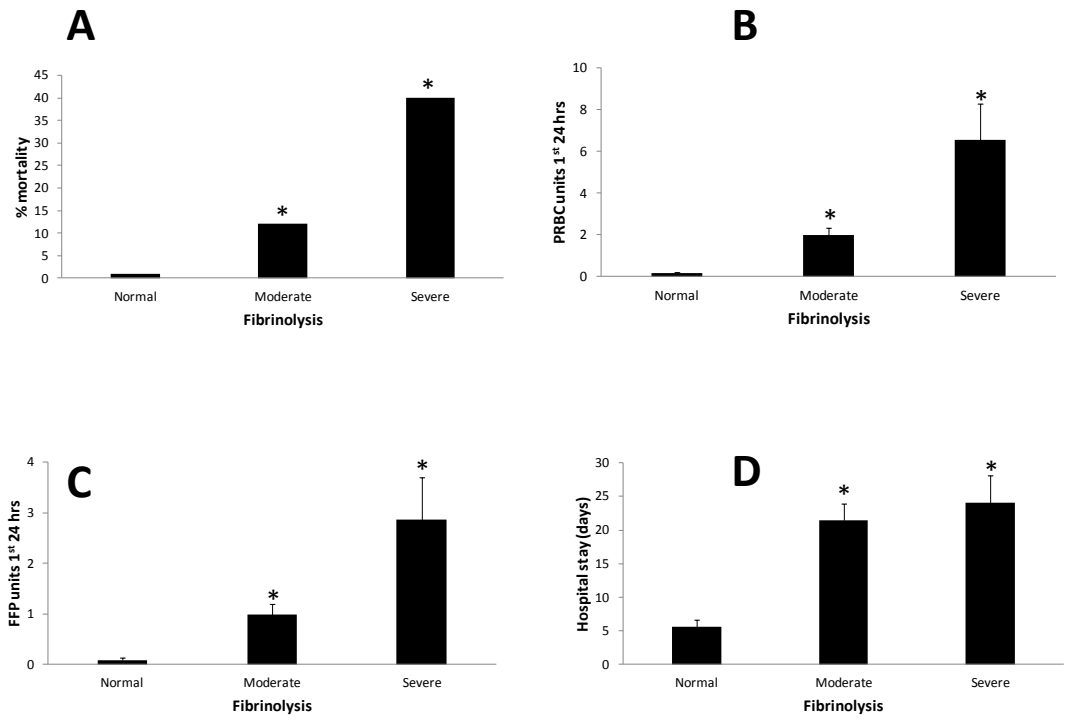


Figure 3.4 Outcomes associated with moderate and severe fibrinolysis

A: Significantly higher mortality in Moderate (12%, p<0.001) and Severe fibrinolysis (40%, p<0.05) vs normal (1%).

B: Moderate and Severe fibrinolysis is associated with higher PRBC transfusion (Moderate vs normal and severe vs normal p<0.001 both groups).

C: Higher fresh frozen plasma (FFP) requirements in Moderate (1.0 units) and Severe (2.87 units) fibrinolysis (Moderate vs normal and severe vs normal p<0.001 both groups).

D: Hospital length of stay in survivors is increased Vs normal (99 survivors**,** 5.7days) in Moderate (145 survivors, 21.5 days), and Severe (9 survivors**,** 24.1 days) fibrinolytic groups (*p <0.001). Values are % or mean +/- 95% confidence interval.

3.4 Discussion

We have identified that FA, as measured by increased levels of PAP, tPA and D-Dimer, occurs in almost two thirds of trauma patients, and the degree of FA is associated with increasing injury and with significantly worse transfusion requirements, morbidity and mortality. This high rate of FA has not been identified previously, but it is consistent with previous work(30), and the efficacy of tranexamic acid in reducing mortality in bleeding trauma patients(34).

Moreover, more than 90% of FA in trauma patients is not detected by the current definition of TEM hyperfibrinolysis. Six in every ten patients presented with moderate FA, but only 1 in 20 showed TEM hyperfibrinolysis. We have shown that increased FA occurs without visible clot lysis by thromboelastometry, despite similar levels of D-Dimer generation as in those patients with TEM hyperfibrinolysis; and that patients with even moderate FA have significantly increased transfusion requirements and other adverse outcomes. The observed FA was not a consequence of hypothermia (at least at levels seen in our study (20)). FA is therefore a real pathophysiological entity with important consequences for trauma patients. This is of concern in the pragmatic management of bleeding trauma patients. The group with moderate FA are larger and account for more deaths than those with TEM hyperfibrinolysis, and would have potentially benefited from the use of antifibrinolytics.

It is unclear under what circumstances TEM detects fibrinolytic activity. It is possible that TEM is simply insensitive until FA reaches a certain threshold. Free plasmin is rapidly hydrolysed and has a very short half-life. In the TEM cup, free tPA is required to generate new plasmin for clot breakdown. This was only seen when tPA levels were nearly 5 times normal and alpha-2 antiplasmin levels were significantly reduced to below 75%. TEM hyperfibrinolysis may only occur when there is insufficient antiplasmin to block the action of all free plasmin generated in the sample well. Certainly the finding that the addition of

aprotinin, a stoichiometric inhibitor of plasmin, to the TEM cups improved the TEM ML trace of those with TEM hyperfibrinolysis, but did not alter the traces of others with FA, suggests that generation of free plasmin is necessary to produce the TEM hyperfibrinolysis. Further research is required to examine this and identify methods to increase the sensitivity of TEM.

There are a number of limitations to our study. Although we have identified that trauma patients have increased FA and that the degree of activation is associated with increased transfusion requirements and worse outcomes, we have not shown that these levels are associated with an increase in clot breakdown or fragility. We used D-Dimer levels to measure the overall results of fibrinolysis, but D-Dimer levels are a marker of prothrombotic fibrin generation as well as fibrinolytic activity. We witnessed significantly higher levels of PF1+2 in the 'severe' group and it is not possible to separate the effects of these two processes based on D-Dimer levels alone. The PAP levels probably represent a more robust marker of the activity of the fibrinolytic system itself. We did not investigate the value of the various stimuli that drive the generation of FA and these mechanisms will require further basic and clinical research. Finally, although TAFI did not appear to be a major factor in the systemic regulation of fibrinolysis it may have an important role in intrinsic clot stability and its role needs further elucidation.

In summary, we have identified FA in the majority of trauma patients and that the degree of FA is associated with significantly worse outcomes in terms of transfusion requirements, mortality and morbidity. These findings provide a logical explanation for apparent paradox that antifibrinolytic therapy appears to have broad application in trauma and elective surgery and yet that massive near-fatal trauma is required to produce detectable changes on a TEM. The discovery of FA in two thirds of trauma patients presents new opportunities

for the development of diagnostic modalities and targeted therapeutic intervention to improve outcomes after trauma.

Chapter 4

The relative contributions of Plasminogen Activator Inhibitor-1, Thrombin Activatable Fibrinolysis Inhibitor and Factor XI in fibrinolytic activation

4.1 Introduction

The importance of fibrinolysis as a mechanism in ATC is now well established. CRASH-2, a large scale pragmatic trial with over 20,000 subjects has shown the benefits of antifibrinolytic therapy in trauma(34). At the other end of the scale smaller studies have shown the coagulation protein changes that suggest that fibrinolytic activation is responsible as a major part of the derangement seen in ATC (Chapter 3 above). Brohi and others have also shown previously that fibrinolysis is an important mechanism and alluded to some of the proteins involved(25,30). Two main inhibitors of fibrinolysis have been described in the literature. PAI-1 and TAFI. Factor XI has also been implicated in the stabilisation of clot and curtailing of fibrinolysis(55). The relative contributions of these proteins in modulating fibrinolysis is not clear.

4.1.1 Plasminogen Activator Inhibitor 1 (PAI1)

PAI-1 is a single chain glycoprotein released by many cell types including endothelial cells, monocytes, macrophages, platelets, hepatocytes, as well as adipocytes(56). It is one of the main inhibitors of plasminogen activation. Its main action is the inhibition of tissue-type plasminogen activator (tPA). This is achieved by binding in complex with tPA and preventing it from converting plasminogen to plasmin(57). The role of aPC in anticoagulation by inhibiting the factor V and VIII pathways is well known but its antifibrinolytic effects, although established for some time(28,58), are less well understood. Associations have been drawn to suggest that PAI-1 inhibition by aPC is an important mechanism in trauma(30). But other groups have demonstrated associations suggesting that overwhelming tPA release is the main driver for fibrinolysis with little effect from PAI-1 inhibition(59). Understanding this mechanism further may point to targets for intervention.

4.1.2 Thrombin Activatable Fibrinolysis Inhibitor (TAFI)

TAFI (also known as procarboxypeptidase B) is a glycoprotein that is synthesised in the liver. It is activated by thrombin but it has been shown that thrombin-thrombomodulin complex speeds up TAFI conversion to activated TAFI (TAFIa)(60) by up to 1250 fold. TAFIa acts as an inhibitor of fibrinolysis by binding to fibrin and cleaving it's lysine and arginine residues. This reduces its co-factor function in plasminogen activation and also makes fibrin more resistant to cleavage by the tPA-plasmin complex(61,62). Control of this mechanism could indeed be a modulating factor in control of fibrinolysis. Indeed animal studies have shown that inhibition of TAFIa by potato carboxypeptidase inhibitor (PCI) does promote fibrinolysis in vivo(63–65). In contrast, from human observational data in trauma, it has been postulated that the role of TAFIa is not clinically relevant(30). This relationship needs further examination.

4.1.3 Factor XI (FXI)

The role of factor XI in modulation or indeed control of fibrinolysis is not immediately clear. Historically it had been labelled in the intrinsic pathway of coagulation. In the current understanding of the cell based model(66) it forms part of the amplification and propagation phase of coagulation and seems to have no direct input into fibrinolysis. However, observations in subjects with FXI deficiency have noted that bleeding, when it occurs, does so from sites of tissue with enhanced fibrinolytic activity(67,68). Furthermore, in vitro studies have demonstrated that inhibition of FXI promotes fibrinolysis(55,69). Von Dem Borne and colleagues have shown that in a system of activation by tissue factor (tF) where lysis was initiated by tPA, the addition of FXIa (activated FXI) inhibiting antibody improved lysis almost two-fold compared to addition of buffer(55)(fig 4.1). This suggests that the presence of FXI attenuates fibrinolysis and its neutralisation promotes lysis. An in vivo rabbit model has demonstrated a similar findings(64). Importantly in this study, blocking TAFI or FXI had similar effects. Moreover there was no additional effect from

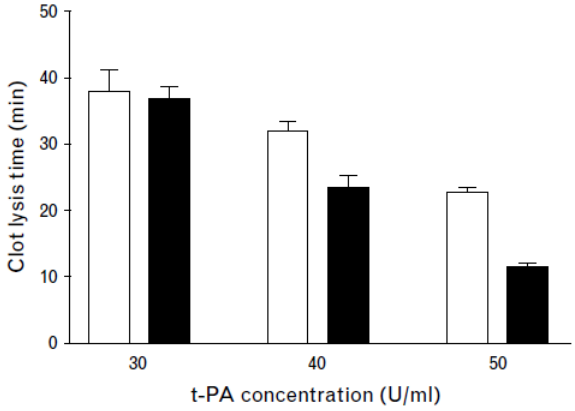


Figure 4.1 Presence of FXI attenuates clot lysis.

Clot lysis time with differing concentrations of tPA to initiate lysis. Open bar with added buffer, closed bar with addition of FXIa inhibiting antibody. Reproduced from Von Dem Borne et al(55)

blocking both simultaneously leading the authors to postulate that the effects of FXI on sustaining clot are mediated indirectly via the TAFI pathway.

Our aim with this work, therefore, is to understand the relative contributions of PAI-1, TAFI and FXI on curtailing fibrinolysis in humans in a trauma setting.

4.2 Methods

4.2.1 Study Design

This was an observational cohort study nested in the ACIT2 trial already described above. The patient group was the same as that in chapter 3 as were the recruitment and blood sampling processes. Data collected for the above study was used in this study but analysed in a different way as detailed below.

4.2.2 Blood sampling

Blood sampling had already been carried out as for ACIT2 above. Specifically for this aim we were interested in tPA, PAP, PAI-1, TAFI, TAFIa, FXI and D-Dimer. These tests were used to investigate the fibrinolytic pathway in more detail.

4.2.3 Data processing

Once data had been collected and all blood results collated special subsets of the data were created. Since we needed to understand the effect of the 3 above coagulation factors on fibrinolysis specifically the data was split in such a way as to identify those patients in

whom there was a significant potential for fibrinolytic activation. Thus the patient cohort was initially split into quartiles of tPA. The highest quartile was taken as the group of interest as clearly these patients would have the greatest fibrinolytic potential. Within this group quartiles of PAI-1, FXI, TAFI and TAFIa were created. The levels of PAI-1 and PAP were compared in this group. FXI activity was also compared to PAP. As TAFIa does not act on the Plasmin, TAFI was instead analysed in the context of D-Dimer; a downstream measure of fibrinolysis. TAFIa was also examined compared to D-Dimer in the lowest quartile of tPA to understand if they had any effect on fibrinolysis where there was minimal and not overwhelming fibrinolytic potential.

4.2.4 Statistical analysis

All the data processing and statistical testing was carried out in MATLAB (Mathworks, Natick, MA, USA). The Kruskal-Wallis test was used to compare the groups where more than two group comparisons were required. No assumption was made about the distribution of the variables (parametric or otherwise). A p value of $<0{\cdot}05$ was chosen to represent statistical significance throughout.

4.3 Results

303 patients were recruited in total for this study as part of ACIT2. 296 patients had tPA measured. Therefore when they were split into quartiles 74 patients remained in each quartile. Summary data for the quartiles of tPA is shown in table 4.1. The normal range for the Asserachrom tPA ELISA in humans is 2-12 ng/ml. The highest quartile of tPA had a range of values for tPA from 13-167 ng/ml. As tPA levels rose PAP levels rose also, and as PAP levels rose D-Dimer also increased (fig 4.2A and B). This demonstrates that where there was high fibrinolytic potential (as evidenced by tPA) fibrinolysis was indeed occurring (as evidenced by PAP and D-Dimer).

Table 4-1 Quartiles of tPA - ISS; Injury severity score, INR; international normalised ratio, ROTEM CA5; mean clot amplitude at 5 minutes on ROTEM, ROTEM MCF; mean maximum clot firmness on ROTEM, 24 hour PRBCs; mean RBC transfusion in first 24 hours, 24 hour FFP; mean FFP transfusion in first 24 hours.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Base Deficit (mmol/l)	0.26	0.88	3.08	4.82
ISS	10	14	15	22
% Penetrating trauma	27%	20%	26%	11%
Age	30	37	39	42
% Male	78.3	85.1	86.5	78.4
INR	1.1	1.1	1.3	1.2
ROTEM CA5 (mm)	43	43	42	41
ROTEM MCF (mm)	59	59	59	57
24 hour PRBCs (units)	0.14	0.30	1.57	4.11
24 hour FFP (units)	0.08	0.12	0.72	2.04
Ventilator free days	27.2	26.9	26.8	26.2
Mortality rate %	2.7	4.1	12.2	17.6

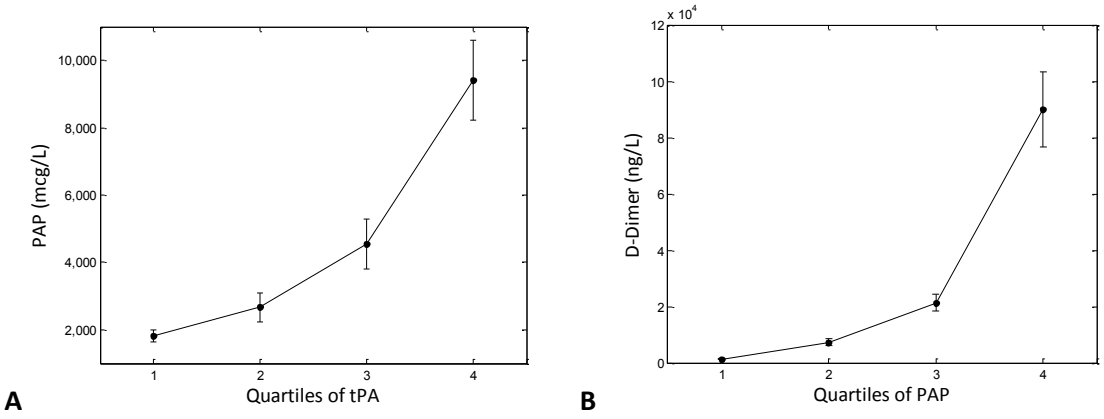


Figure 4.2 tPA and D-Dimer

A)Relationship between tPA and PAP

B) Relationship between PAP and D-Dimer

Points plotted are mean and error bars are standard error of the mean.

4.3.1 PAI-1

The normal quoted range given for the Asserachrom PAI-1 ELISA was 4-43 ng/ml. PAI-1 was also split into quartiles to understand its relationship better. When PAP was compared to quartiles of PAI-1; PAP levels were low in the lowest quartile of PAI-1 and rose as PAI-1 levels went up but in the highest quartile of PAI-1; PAP was low again (fig 4.3A). This relationship is not easy to explain. We then looked at this relationship only in the highest quartile of tPA (where there was significant fibrinolytic potential). In this group overall PAP levels were higher as would be expected when tPA was high. In the highest quartile of PAI-1 PAP was significantly lower compared to other PAI-1 quartiles, suggesting that high PAI-1 levels may have an inhibitory role on Plasminogen activation (fig 4.3B).

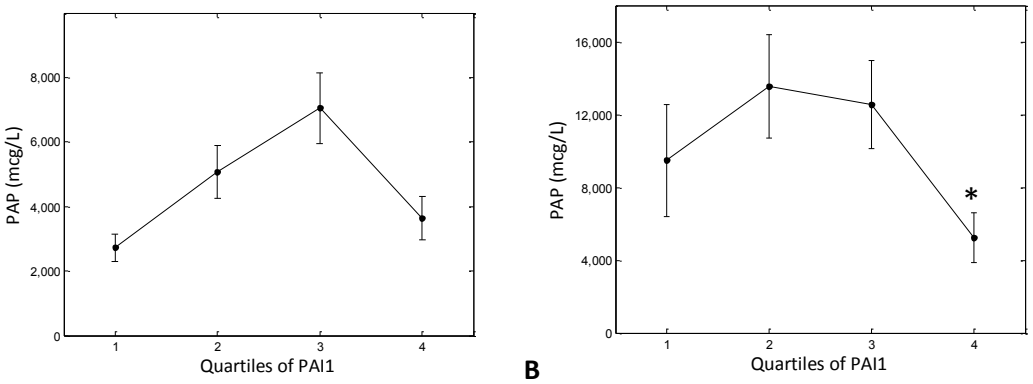


Figure 4.3 PAI-1 and PAP

A) Quartiles of PAI-1 in all patients

B) Quartiles of PAI-1 in highest quartile of tPA. *- significantly lower than other quartiles, p<0.05

Points plotted are mean and error bars are standard error of the mean.

4.3.2 TAFI

The normal quoted range given for the Asserachrom TAFIa ELISA is 8.53 to 22.07 ng/mL. When TAFIa was compared to D-Dimer in the highest tPA quartile the relationship seemed to correlate very well (fig 4.4A). This is counterintuitive as there should be a negative correlation if measured circulating TAFIa was truly inhibitory to fibrinolysis. We went on to examine the relationship in the lowest quartile of tPA to understand if TAFIa could inhibit fibrinolysis where there was much lower pressure for fibrinolysis to occur. In this group also the relationship was similar (fig 4.4B) suggesting that the TAFIa pathway is not a major inhibitor of fibrinolysis in trauma.

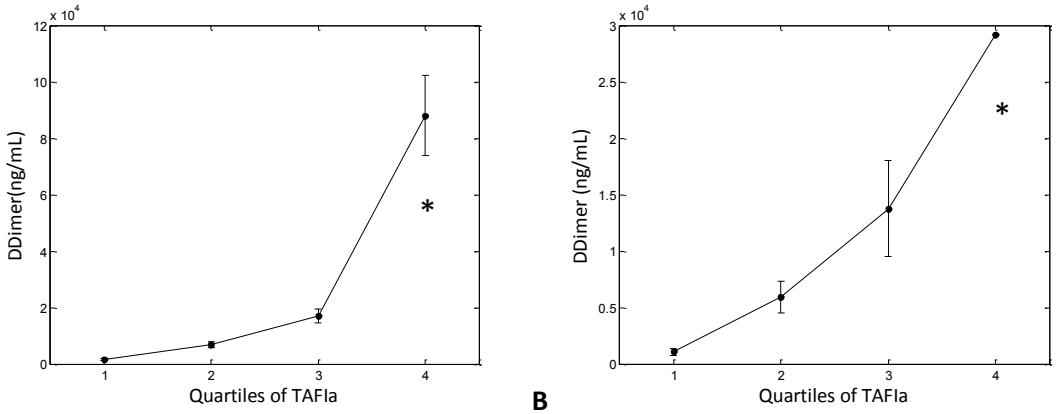


Figure 4.4 TAFIa and tPA

A) Quartiles of TAFI1 in highest quartile of tPA

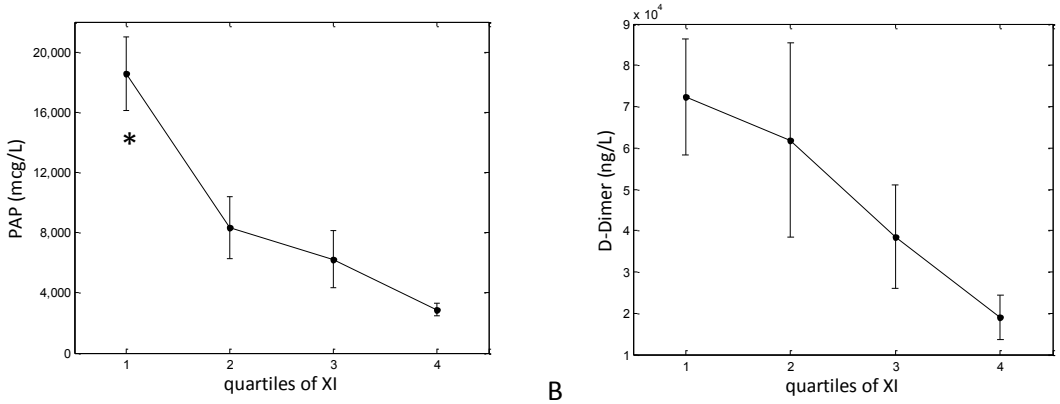
B) Quartiles of TAFI1 in lowest quartile of tPA

***- significantly higher than other quartiles, p<0.05**

Points plotted are mean and error bars are standard error of the mean.

4.3.3 FXI

The quoted normal range for FXI activity is 48-158 iu/dL. In the highest quartile of tPA there is an inverse relationship between FXI activity and PAP levels. Where FXI activity was maintained PAP levels were higher than normal but in the lowest quartile of FXI; PAP levels were significantly higher (fig 4.5A). FXI activity in this quartile ranged from 11 to 84iu/dL with a median activity of 69iu/dL. Indeed this activity is very low. Since the specific mechanism for influence of FXI on fibrinolysis is not known, FXI was also compared with D-Dimer (fig 4.5B). The relationship with D-Dimer may be an important one as there is some suggestion that FXI influences fibrinolysis via the TAFI pathway(55).



A B

Figure 4.5 Factor XI and lysis

A) PAP levels at different quartiles of FXI in the highest tPA quartile *- significantly higher than other quartiles, $p<0.05$

B) D-Dimer levels at different quartiles of FXI in the highest tPA quartile

Points plotted are mean and error bars are standard error of the mean.

4.4 Discussion

The specific mechanisms in acute traumatic coagulopathy and the dominant coagulation system pathways need to be established with further clarity. This will help us to understand the processes involved but more importantly tailor interventions and management accordingly. This work is aimed at understanding some of these pathways.

We have shown that PAI-1 levels where high are associated with lower levels of PAP and therefore fibrinolysis, even in the cohort of patients where there is raised tPA and therefore a significant drive towards fibrinolysis. PAI-1 may therefore be an important control mechanism in fibrinolysis in trauma. The Denver group have suggested that fibrinolytic activation is dependant more on overwhelming tPA release binding free PAI-1 than on PAI1 depletion by aPC(59). Their assertion is therefore that research focus should be kept more on tPA upregulation than on PAI-1. But our work shows that even in the presence of high tPA levels, Increased PAI1 is associated with lower levels of FA, suggesting that PAI1 still has a modulating effect and is not simply overwhelmed. Whether it is depleted by tPA or inhibited by aPC it could still be a target for intervention and control.

The role of circulating TAFI is not immediately obvious in trauma. Animal studies have shown that circulating TAFI can have an inhibitory effect on fibrinolysis in vivo(63–65). But our observations demonstrate no such response. TAFIa is simply correlated well with rise in D-Dimer, suggesting that rises in TAFIa levels are associated with higher levels of fibrinolysis; contrary to its expected role. The clinical relevance of measuring circulating TAFI or TAFIa have been brought into question previously in trauma also(30). In our observations, TAFIa seems to simply be a marker of fibrinolysis rather than a controlling factor. Why this should be is an interesting question. Perhaps the expected mechanism of action may explain this somewhat. TAFIa binds to formed partially degraded fibrin. This binding then curtails further degradation by plasmin and therefore fibrinolysis (fig 4.6) in a competitive manner. But the formation of fibrin is the final step in a mechanism that may

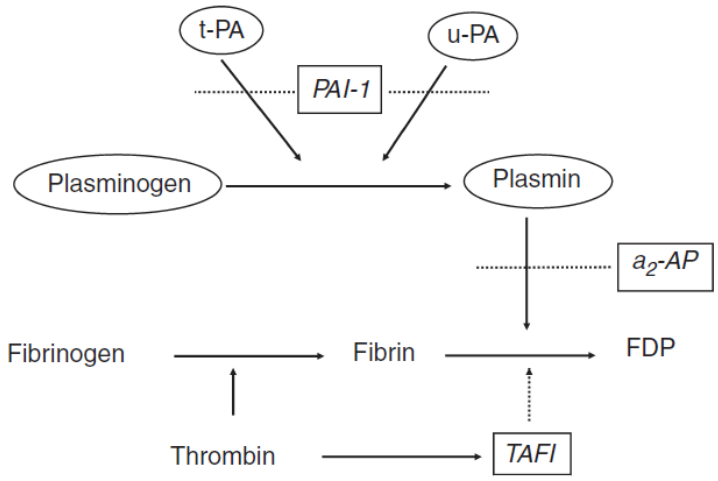


Figure 4.6 TAFI and PAI-1

The mechanism of fibrin degradation and its modulators(57)

simply overwhelm the mechanism by which TAFI works. In massive blunt trauma where clot formation may not be localised to a single locus, this may be particularly so.

It has also been shown that as well as circulating in blood TAFI is also present in platelets(70). This could potentially be an important source of TAFI and may well be the dominant source of fibrinolytic inhibition on a very local level. Of course this would be incredibly difficult to measure or investigate and therefore, at present, understanding the role of TAFI remains challenging.

Plasmin can also activate TAFI (71)by cleaving it to form activated TAFI (TAFIa), but this mechanism is more difficult to understand as it seems that plasmin also inactivates TAFIa and this might well be a modulating mechanism for Plasmin to promote fibrinolysis. In our cohort of trauma patients we have already shown that PAP levels and fibrinolytic activation is much higher than previously described (chapter 3). This activation of Plasmin may well be responsible for cleaving and inactivating TAFIa and thus negating its role as an important inhibitor in this process.

See also plasmin paper by Marx first paragraph(71). Maybe TAFI only blocks the positive feedback loop so its effects in trauma are minimal

Our observations regarding FXI and its relationship with fibrinolysis mirror some of the in vitro work closely. In our cohort (in the highest quartile of tPA) FXI activity, where relatively well preserved (quartiles 2,3 & 4 of FXI), was associated with moderate levels of PAP. But in the lowest quartile of FXI the levels of PAP were observed to be very high (between 16,000 and 20,000 mcg/L – Fig4.5A). FXI activity in this group was much lower than other groups (range 11-84iu/dL). This is similar to the in vitro data where clot lysis occurred rapidly when FXI was inhibited(55)(Fig4.1). These data would suggest that depletion of FXI promotes fibrinolysis, rather than high levels of FXI attenuating it. The mechanisms for this are not clear.

FXI mediated thrombin generation is an important mechanism of clot propagation(72) once initial tissue factor burst has occurred. In-vitro data have shown that this clot propagation and FXI dependant thrombin generation is responsible for inhibition of fibrinolysis(73). Although there has been suggestion that this mechanism works by promoting thrombin generation and TAFI activation(74) our observations above would suggest that this may not be the dominant pathway since the concentration of circulating TAFI does not seem to be related to inhibition of fibrinolysis. Certainly more evidence (probably in vitro and in vivo) is required to elucidate the specific mechanism further.

Our observations in this study do mirror and support some of the previous in vitro and in vivo work. And this work is part of an increasing body of evidence from observational cohorts of trauma. Historically, this type of work has been invaluable in beginning to understand the mechanisms involved in ATC and TIC and relate them to evidence from lab and basic science research. Unfortunately this type of data can only draw conclusions on associations and not on causation and molecular mechanisms. We have also not taken into

account here the possible contribution of platelets to the whole process. As stated above, platelets may be an important or even dominant contributor to the role of TAFI. It may certainly play a role in PAI-1 and FXI modulation also. Certainly since the better understanding of the cell based model of coagulation the importance of the role of platelets has led to much research interest and this will continue. In this work, however, it is difficult to include platelets in a meaningful analysis.

In addition one of the further challenges (if not main difficulty) in this type of observation is the large number of possible variables that may confound findings. For example the FXI activity may well be influencing other factors. We do know that there is a pathway to relate FXI to fibrinolysis so it is appropriate that we compare these but it is entirely possible that these are simple correlations and other confounders need to be addressed. With so many factors involved in the coagulation pathway, even when the mechanism is not under stress (such as in trauma), it is challenging to fully appreciate the systems involved. In a situation such as trauma this becomes even more challenging.

We need to explore other ways of understanding and analysing this data and one possible route is to leverage exploratory data analysis techniques which are becoming more ubiquitous when looking at large bodies of data. In the following chapters we will use these newer techniques to try and improve our understanding further.

Chapter 5

Exploratory data analysis techniques and methods

5.1 Introduction

The traditional scientific method is based on making observations, perceiving trends, formulating hypotheses, collecting data and then analysing that data against said hypotheses. The hypotheses are based on the perceived trends and the observations made at the outset. This is an effective method and essentially the backbone of most scientific and medical research historically. In the modern world where we have tools to collect, store and analyse large amounts of data, this paradigm is being challenged. Part of the problem with this type of process is that our own biases are introduced even from an early stage. This may not be a bad thing since our observations and perceptions of trends may well be quite accurate. The human brain is capable of classifying trends effectively and over hundreds of years the scientific method has served us very well indeed. But is there a different way? When we formulate a hypothesis, immediately we introduce a bias into the way we look at data. Perhaps if we had looked more openly at everything the dataset had to offer we may arrive at important conclusions which may not have been otherwise obvious. This is where exploratory data analysis can be helpful. It is particularly helpful in very large datasets which we are now capable of generating. These large datasets may not lend themselves to being easily observed or in fact easily examined by traditional techniques. Exploratory data analysis should not be seen as a replacement to the scientific method. It can be used as an adjunct and at times a precursor to gain an initial understanding of data.

An advantage of using exploratory data analysis techniques is that they can help to simplify or reduce the dimensionality of data. For example, if we take a simple process where a few variables are involved in a mechanism and we are unsure which are important and how they relate to each other, one way to begin analysing the data may be to plot x against y and look at the result. We may even be able to add a third variable to the plot to understand how the three relate to each other. Beyond three variables simultaneously, in

conventional statistics and data analysis we couldn't visualise the relationship. Even without the need to visualise the relationship it may be difficult to understand it. Multiple regression analysis is sometimes used to explore the relationships between many variables. Even this is limited to essentially outputting an equation and finding best fit parameters. And in reality, multiple regression still only analyses the relationship between lots of variables and how each relates to only one output variable. In order to understand and explore more variables at the same time we need to utilise more complex techniques. Techniques which look at very large numbers of variables and aim to reduce them are known as dimension reduction techniques. Principal component analysis is an example of dimension reduction. Techniques which look at very large numbers of observations (which may also have many variables) and look to reduce these or group them are broadly known as classification or clustering techniques. Both these types of analysis are used to explore large datasets and can be set up to do so in an unbiased manner.

5.1.1 Clustering

Clustering is a technique in which observations are classified into groups based on their similarity to other observations. Similarity measures are calculated across all variables simultaneously in order to classify any given observation. This is the main power of the technique. Various types of clustering techniques exist and even within each type further subcategories and specialisations have been produced over time to work with specific types of data sets. The most common use of clustering in the medical sciences is to classify gene expression data. This can be done at both DNA and RNA levels. Increasingly this type of analysis is being used to understand cancer biology and to tailor interventions appropriately. Examples in breast(75,76) and lung tumours(77) exist but the technique is being more widely used to good effect.

Types of clustering

As stated previously multiple clustering methods have been defined. The main types and those in commonest use include hierarchical clustering, K-means clustering and density based clustering. They each have their advantages and disadvantages but these may be specific to types of dataset.

5.1.2 Hierarchical clustering

This is perhaps the most straightforward type. It is an example of unsupervised learning. That is, it requires no specific starting conditions and once set up and will cluster without further input from the user. In this respect it minimises bias as much as possible. It results in good visualisations of output and therefore is helpful in giving a good overview of the data visually so giving us a better understanding(78). It is a relatively simple technique and therefore easily accessible. Most bioinformatics programmes will be able to handle this type of clustering. Its disadvantage is as a result of its simplicity. Because complex calculations are not being carried out, a lot of calculations are required and therefore it is very computationally expensive and inefficient.

5.1.3 K-means clustering

This type of clustering works in a very similar way but requires the user to set the number of clusters or the 'k' prior to starting. It also needs further inputs to begin clustering and so introduces a potential for bias. Having said this, many strategies and mechanisms exist to minimise this bias of human input. K-means is a more complicated method overall than hierarchical and therefore is less accessible to those not proficient in these methods. Importantly it is also a stochastic method, that is to say it is much more statistically based and even inputting the same 'seed' data may reveal subtly differing outcomes. In very large datasets this may not be an issue but in smaller datasets (like ours) this certainly would give one less confidence. Compare this to hierarchical clustering which is deterministic. This means that if the same process is run then the same outputs from the algorithm will result. This makes results reproducible in smaller datasets which may be a requirement. Many

different variations of K-means clustering exist and are in common use for various different applications(79).

5.1.4 Density based clustering

This type of clustering has the advantage of being computationally very efficient. This lends itself much more to use in very large datasets. It also has the benefit of dealing much better with outliers than the other methods. However, many more inputs are required to run the algorithm and much more specialist knowledge and indeed software is required. It is also not as easy to interpret the outputs visually. All this means that it is not as easily accessible to less experienced data handlers who are working on smaller datasets. Indeed, one could argue that if datasets are not very large this type of clustering holds minimal advantages.

For the purposes of our analysis hierarchical clustering is more than adequate and has the advantage of being easy to access and reproducible across smaller datasets with good visual outputs for quick referencing and interpretation.

5.2 Methods to prepare data for clustering

5.2.1 Patient recruitment and data acquisition

The patients for this part of the study were recruited in the same manner as those described above. As well as the first 323 original ACIT2 patients, recruitment continued and eventually included the patients and samples recruited by our European partners. In all, the data that is presented here comprises patients recruited from January 2008 until July 2014. It should be noted that study recruitment continues to the present time. All patients were recruited and consent obtained according to the same criteria detailed above, with local variation based on each centre's own ethical approval. The timescale for blood sample collection and processing was the same everywhere irrespective of local centre variation in other administrative aspects. Once the samples had been processed and stored (in line with the SOP detailed in chapter 2, section 2.4/fig2.1), they were eventually returned to our centre and sample analysis undertaken when funds and sample kits were made available. All centres also collected the same patient data as detailed above in section 2.7. This included data on patient demographics, injury characteristics, IV fluids and blood transfusion in the first 24 hours and outcome data.

All of the above data acquisition resulted in a large data set comprising various types of data. For ease of description later on I have split these into discrete data sections;

Demographic and injury (D&I) data

All the data obtained at admission describing the patient demographics, injury characteristics, (AIS and) ISS data worked out by our TARN (trauma audit and research network) data collectors. I have also included initial lab or bedside tests under this category such as blood gasses (lactate/base deficit) and admission full blood count etc. This also included detailed data on fluids infused prior to first blood draw.

Biological data

All blood samples collected and analysed by ourselves resulting in ELISAs and all blood samples handled by our laboratory to give numbered coagulation factors and a few other coagulation proteins as described in detail in chapter 2 and summarised in tables 2-3 and 2-4. It should be noted here that the decision was made to not proceed further with testing of TAFI and TAFIa (ELISAs). This decision was based on various factors. Primarily as a result of our work described in chapter 3 and 4 and also undertaken previously(30). Given that there was a lack of signal from the circulating TAFI and TAFIa measured in these studies, and, the significant cost both in terms of time and money to analyse, what would have been, around 2,000 samples (as a minimum), further analysis of these ELISAs was not undertaken.

ROTEM data

ROTEM data was analysed across all sites based on the SOP previously described and in line with the company's own guidelines.

Resuscitation fluids (RF) data

This was detailed data collected on volumes and types of intravenous fluid required in the first 24 hours of admission after the initial blood draw. This did not include the fluids given that are already accounted for in the D&I data described above.

Outcome data

All data collected on outcomes such as 28 day outcome (discharge or death), total LOS, critical care days, ventilator free days, vasopressor days and renal replacement therapy (RRT) days.

5.2.3 Data to be imported for clustering

The aim for this project was to use clustering to understand the coagulation system derangements in trauma. The most important dataset in this respect is the biological dataset. We wanted to define the different derangements based only on the biological data for a number of reasons;

- The biological dataset contains all the coagulation protein data. This is essential to our understanding of coagulation and coagulopathy.

- The biological dataset comprised continuous variables which are much more suited to analysis by clustering. The other datasets comprise largely discrete and categorical data. Some of these are even binary data. Although it is possible to cluster these data it is not ideal and much more complex as a method.

- The other datasets are likely to have more missing data. This is an inherent problem in collecting clinical data and can be a difficult problem to deal with.

5.2.4 Data cleaning

The process for importing a clean dataset into the clustering algorithm needed to be rigorous. I have detailed below the stepwise data process undertaken to achieve this.

5.2.5 Dealing with missing data

In order to successfully import a dataset into a clustering algorithm one must have a complete dataset. In our dataset missing values occurred mainly where there had been either a problem with test itself or where there was insufficient sample to process every test. Where blanks in data set occur a few options are open to us in order to proceed(80). Deleting the whole sample from that subject is a possibility. The other option is to replace the missing value with a calculated value in some way. There are various methods of calculation from as simple as using the mean for that particular dataset to much more complicated equations to replace values(81). These methods are all broadly known as imputation. Statistical model based methods are also used, for example using Bayesian network prediction to replace the data. Although the more sophisticated methods are certainly better than simple imputation (using means for example), nevertheless they all have the potential to introduce bias into the data and thereby reduce the accuracy of

analysis and therefore conclusions derived. For this reason the missing data in our dataset was dealt with by deleting the subject and using complete-case analysis method only.

5.2.6 Sorting upper and lower limit values

There were other instances where data was not missing but was given as a high or low level. This occurred with our ELISA data. Where a sample result returned as 'greater than' the highest interpolatable value given by the manufacturer of the ELISA kit, we attempted to redo the test with a half dilution sample to get an accurate reading. If it was still above the upper range this was noted. Where a sample appeared to be below the range we were unable to dilute or concentrate this further and so this was simply noted in our overall database. When coming across these values in the cleaning stages they were replaced with the highest or lowest value attributable for that test batch. Although these may not be accurate estimates this practice assumes the value is either very high or low, which is biologically true and therefore does not unduly bias the result of analysis or interpretation.

5.2.7 Dealing with outliers

On the whole outliers were not an issue in the biological dataset. Many time values that seemed like outliers were simply very high or low values. All raw data was plotted to visualise the possibility of outliers, and for all samples (except one), no deletions were required. The one exception to this was a result for Factor VII. The normal range for this test was given as up to 50-150 iu/dL. One value returned a result of 7045 iu/dL. The next highest value in any FVII assay was 1245 iu/dL. As there was enough sample remaining the test was repeated but revealed a similar result. After consultation with our lab manager, who supervised this analysis on the Sysmex lab machine, we could not arrive at a reasonable conclusion as to why this could be, particularly since the remaining factor and ELISA values for the same subject were not outliers. Initially in preliminary clustering this subject was left in the analysis since the sample had been repeated and the same value returned. But on reflection leaving in such a high value was unduly transforming the

remaining FVII values in our data process and therefore destroying the variability seen in the FVII dataset. It was therefore removed before the final analysis.

5.2.8 Thrombomodulin ELISA

As described in chapter 2 (section 2.6) our sample processing was run in two batches; upto patient 324 in the first batch and then all remaining samples in subsequent batches. All tests were reproducible between batches with the exception of the thrombomodulin ELISA. This was no longer available by the second batch and therefore a different kit was sought. The characteristics for the new kit were quite different (table 2-4). It would have been impossible to retrospectively repeat the first batch of samples on the newer kit. At the same time excluding one or the other would significantly reduce our sample size and available data. Therefore both batches have been left in. It will not be possible to draw inferences about the raw values, however, given the normalising process (see below) even having different ranges of the nature seen in these two ELISAs does not exclude the whole TM dataset from the clustering algorithm.

5.2.9 Normalising data

The hierarchical algorithm used will cluster data based on similarity between different tests and also between different subjects based on similarity. For the similarity to be accurately measured the units of each data point must be similar. Before importing the data into the algorithm it must, therefore, be normalised. This ensures that the only thing affecting the clustering is the pure variability within the dataset and not units within or between datasets. There are various methods of normalising data. They are broadly similar and give similar results(82). However, we did note Milligan and Cooper's observation that using the z-score (a commonly used method in some clustering algorithms) can sometimes remove some of the variability in the dataset. Therefore the following normalisation formula was used:

$$(\boldsymbol{R}-\boldsymbol{x})/_{\boldsymbol{X}}$$

Where R = the mean of the normal range given by the manufacturer of that test

Where X = the range of values in a given dataset (ie max-min)

Where x = the observed value to be normalised

This transformation was carried out separately within each sample for the batches before and after sample 325. Dividing by the range meant that all datasets had a range of 1 and were centred around R. When the two batches were then combined the values were now comparable. This normalised biological dataset could be finally be imported into the clustering algorithm.

5.3 Methods in Clustering

Hierarchical clustering works in 3 stages

1. It seeks to quantify the similarity between observations by measuring the mathematical distance between observations.
2. It uses a linkage algorithm to determine which observations are similar based on the mathematical similarity between them
3. Visualisation and 'cutting' of the clustergram and dendrogram to reveal the final clusters

5.3.1 Distance metrics or proximity measures

In order to achieve the first stage we need to have some way of calculating the difference between observations and this is known as the distance metric or the proximity measure. To calculate a distance between two observations in two or even three dimensions is straightforward. Each dimension here is a variable. In order to calculate the distance in more than three dimensions requires more complex mathematical solutions. A number of proximity measures exist in order to calculate these distances. These measures are split into quantitative or qualitative. Examples of quantitative are Minkowski, Euclidean, Manhattan block, Chebychev and squared Mahalanobis distance. Qualitative include Pearson correlation and Spellman rank correlation. It is widely accepted that the default proximity measure is Euclidean distance(83), and for most applications it performs very well.

5.3.2 Agglomerative or divisive clustering

To achieve the second stage of discerning similarity (or difference) the clustering method can use a number of possible algorithms. These are known as 'linkage algorithms'. Once the distance metrics have been calculated between each observation, the clustering algorithm will then apply the linkage algorithm of choice to create the cluster. This can be done in two possible ways; agglomerative or divisive.

Agglomerative hierarchical clustering is a 'down to up process' or sometimes referred to as 'leaves to trunk'. If we assume the number of observations in a complete cohort is *n*. This type of clustering stars with *n* clusters and combines a cluster with its nearest neighbour using the linkage algorithm to guide it. It then keeps joining clusters to other clusters until the number of clusters = 1, i.e. all observations are joined into a single cluster.

Divisive clustering is the opposite. It is a trunk to leaves process where the algorithm starts with one cluster that contains all *n* of the observations. It gradually separates the 1 into more than one and subsequently many cluster, again based on the linkage algorithm. This is continued until there are *n* clusters. Divisive clustering is much more complex and computationally expensive and therefore was rarely ever used. In fact the software suite I have used in this project defaults to agglomerative clustering and does not have a built in function for divisive clustering.

5.3.4 The dendrogram

Hierarchical clustering algorithms output two main constructs; the clustergram (sometimes referred to as the heat map) and the dendrogram. The heat map is a graphical representation of the whole dataset with colours for each variable in each subject to represent whether the observation is high or low compared to a given central point. This in itself is an advantageous data analysis adjunct. The true output, however, is the dendrogram. This shows the hierarchy of all the clusters and how they relate to each other. It shows how many clusters are formed and how the separate (or join) and at what level. 'Cutting' this dendrogram at different levels will reveal different numbers of clusters in the dataset (Example dendrogram Fig 5.1). This is again somewhat of an iterative process requiring trial and review.

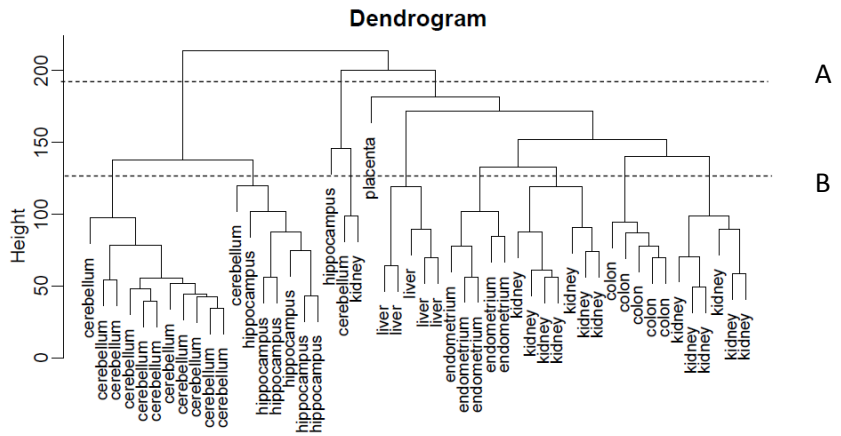


Figure 5.1 Example dendrogram

Cutting this dendrogram at point A will result in 3 clusters whereas cutting at B will give 10 clusters

This does have the potential to introduce bias but coupling this step with the linkage method is crucial. Some linkage methods will reveal a very well set out dendrogram where clusters are easy to discern and another may reveal a very tightly packed dendrogram which separates all levels very closely and so makes it difficult to discern the separation of clusters effectively.

5.3.3 Linkage algorithm

This linkage algorithm itself is the specific algorithm used to decide on each cluster. Let us take the simplest example. Single linkage clustering creates clusters by joining clusters to nearest neighbours. This is calculated by finding the nearest 2 points in any given clusters (Fig 5.2A). This distance is then compared to other nearest clusters and the nearest clusters are joined.

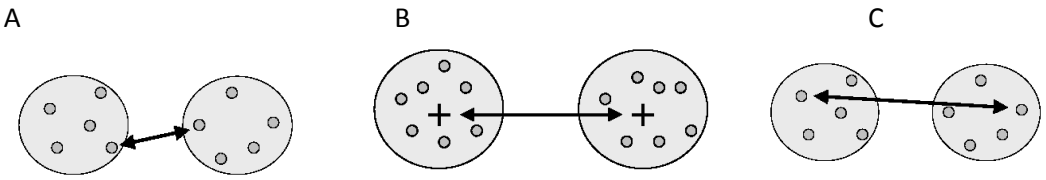


Figure 5.2 Linkage methods

A) Single linkage – nearest neighbour in another cluster

B) Centroid linkage – distance between centroids of each cluster

C) Complete linkage – furthest neighbours in each cluster

(adapted from www.moline.com)

A number of other linkage methods exist. Two others are also shown in fig 5.1. As all datasets are different finding the correct linkage methods is somewhat of a trial and error process. This is an exploratory data analysis after all. However, it is acknowledged that single linkage method performs poorly in almost all situations(83). As it was one of the earliest described methods(84) it is usually included for completeness in almost every software package. In this work centroid, average, median, ward and complete linkage methods were evaluated in our dataset. Prior to acquiring the international dataset, preliminary clustering was run on the same dataset described in chapters 3 and 4. This work was presented as an abstract at the European shock society conference in 2013(85). Working examples from that preliminary project are shown in Fig 5.3 below. Again single linkage was carried out for completeness but its disadvantage is obvious here looking at the dendrogram. Centroid, average and median linkages also show a challenging dendrogram to interpret and separate and any given level usefully. The two most useful methods for this particular dataset were ward and complete linkage. They output dendrograms which appear to be well separated and relatively easy to cut to give useful clusters.

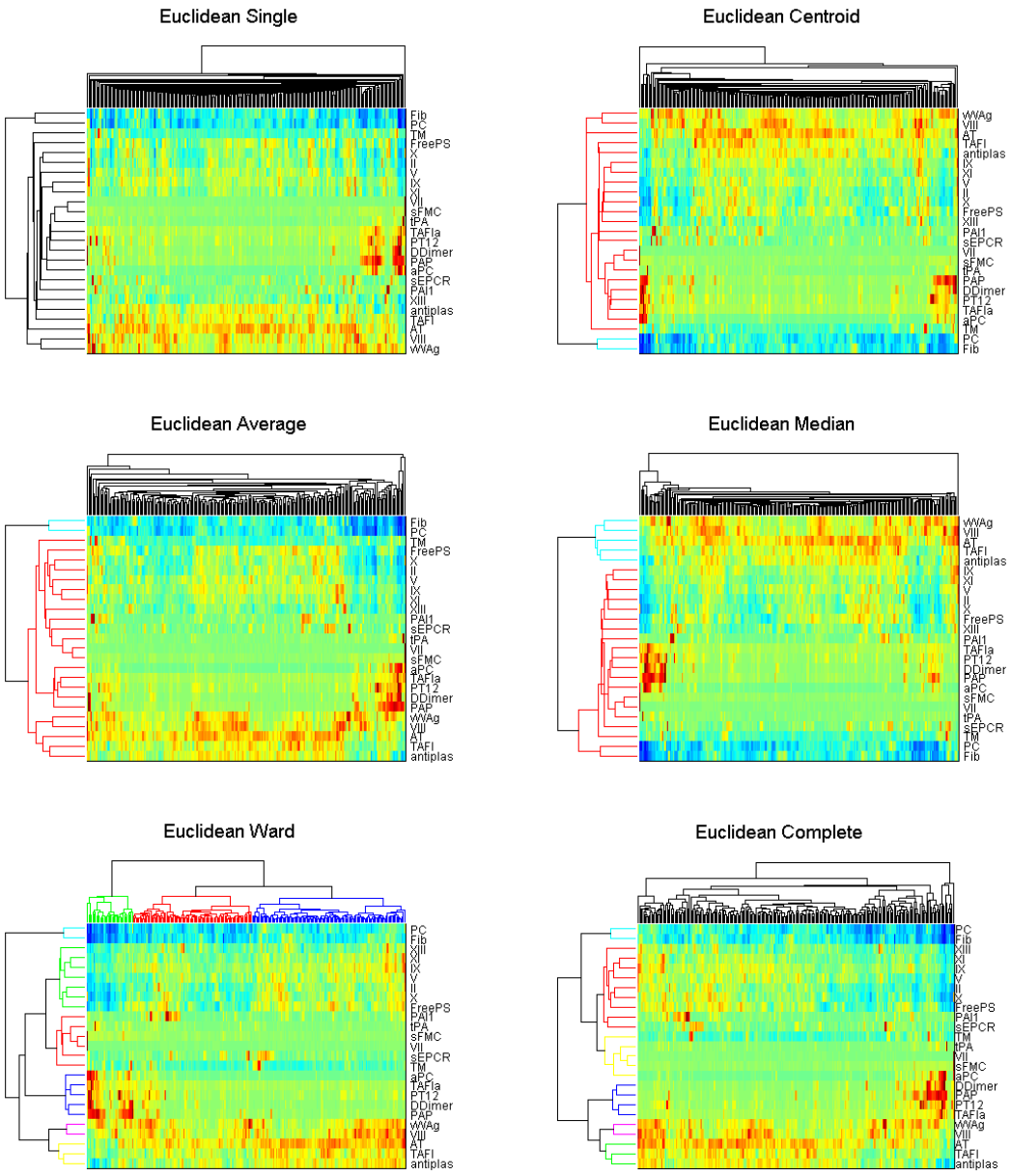


Figure 5.3 Linkage algorithm examples

Examples of clustergrams and associated dendrograms for six linkage methods, all using Euclidean distance metrics. Only Wards Linkage and Complete Linkage seem to perform well on this test dataset

5.4 Data processing and analysis tools

Microsoft Excel 2010 (Microsoft Inc., Redmond WA, US) and MATLAB (Mathworks, Natick, MA, USA) were the two packages used together during the whole process. Data was initially available in various different excel files. These needed to be merged to allow analysis of the complete dataset. Collating and merging such large volumes of data manually often results in transcription errors. The data was therefore imported into MATLAB and its powerful merging tools were utilised to construct the whole dataset. This was then exported back to Excel where the biological dataset was prepared for clustering as described in section 5.2 above. The final normalised dataset was then reimported into MATLAB where the clustering could be carried out using the Bioinformatics Toolbox. The dendrogram could be reviewed and decision on cutting made at this stage. The final clustering information was then re-merged with the complete dataset in MATLAB. The statistics toolbox could now be used to analyse the output from the clustering.

Chapter 6

Using hierarchical clustering methods to classify coagulation system derangements in trauma

6.1 Introduction and aim

The acute coagulopathy that occurs early after trauma is an important yet difficult mechanism to understand. To date work has shown that fibrinolysis and associated mechanisms are responsible for this derangement(25,30 & chapter 3 above). A large randomised controlled trial of an antifibrinolytic agent showed survival benefit(34). Yet the specific derangements are still difficult to fully understand. Moreover, with such a variable patient population and such a difference in the possible initial insults there could be more than one mechanism at work. This degree of variability in presentation poses challenges in understanding and interpretation of trauma data and outcomes. When we do analyse this data we may unduly introduce bias into testing and understanding these datasets. As datasets get larger we may not be able to understand them at all. A need exists to try and understand this degree of variability in an unbiased way.

Hierarchical clustering may provide a good solution to begin to understand these types of data. Clustering has been used ubiquitously in exploration of gene expression data and very large data sets. Very few people have attempted to use it on medium sized datasets in the medical fraternity on non-genetic data. In theory it would provide a good solution to explore a dataset of hundreds of individuals with multiple variables associated with each observation. However, since hierarchical clustering has not been used in this context before, a bespoke solution may need to be sought to achieve this.

My next aim therefore is to utilise a hierarchical clustering algorithm on biological coagulation factor data to help classify the patterns of coagulation system derangement in trauma.

6.2 Study design

Trauma patients were recruited to an ongoing multicentre prospective observational cohort study. Five discrete datasets were collected from each patient. The recruitment and data collection has been described in detail in previous chapters. The data from all European centres was collated. The biological dataset was extracted and appropriately prepared for cluster analysis. This data was imported into a hierarchical clustering algorithm for analysis to classify the different coagulation system derangements.

6.2.1 Statistical analysis

All data were analysed in MATLAB (Mathworks, Natick, MA, USA) utilising its statistics toolbox. Since there were more than two clusters (groups) to compare, the Kruskall-Wallis test was used to compare groups. No assumption was made about the distribution of the groups at the time of testing. The 'multicompare' tool in matlab was used to understand the output of the Kruskall-Wallis test to see which specific groups were statistically different to other groups. A p value of 0.05 was chosen as a threshold for significance. Data are presented below in the form of mean with 95% confidence intervals, or, if they are obviously non-parametric data (such as length of stay), they are presented as median with interquartile range. Charting of non-parametric data is done using box and whisker plots and all other data is charted using mean plots and confidence intervals as error bars.

6.3 Results

1539 patients were recruited from a total of 6 European centres between January 2008 and July 2014. No less than 118 variables were observed for each patient. This resulted in approximately 181,602 data points. Numbers of variables from each dataset were as follows;

- D&I variables 39
- Biological 21
- ROTEM 44
- RF 8
- Outcomes 6

The biological dataset was isolated and cleaned in preparation for clustering. 231 patients were removed as they had 1 or more blank data fields. 78 further were deleted as they had insufficient plasma sample remaining to allow analysis. 1 patient was excluded because of a significant outlier. This left us with 1229 patients. In these patients, 149 data points in the biological dataset were replaced by high or low values for that variable. The biological dataset was separated and imported to the hierarchical clustering algorithm in MATLAB. Once the clusters were defined the complete data set was re-merged with the cluster data and analysis of clusters could begin.

6.3.1 Linkage algorithm

Results were obtained by applying hierarchical clustering to the biological dataset using ward and complete linkage algorithms. The dendrogram for complete linkage appeared to be relatively well separated but had to be cut at various different levels to obtain a reasonable number of clusters. When cut across at the same level it revealed 15 different clusters. On the other hand ward linkage performed well, giving a well separated dendrogram and easily discernible clusters on the dendrogram. It revealed 6 distinct clusters (Fig6.1).

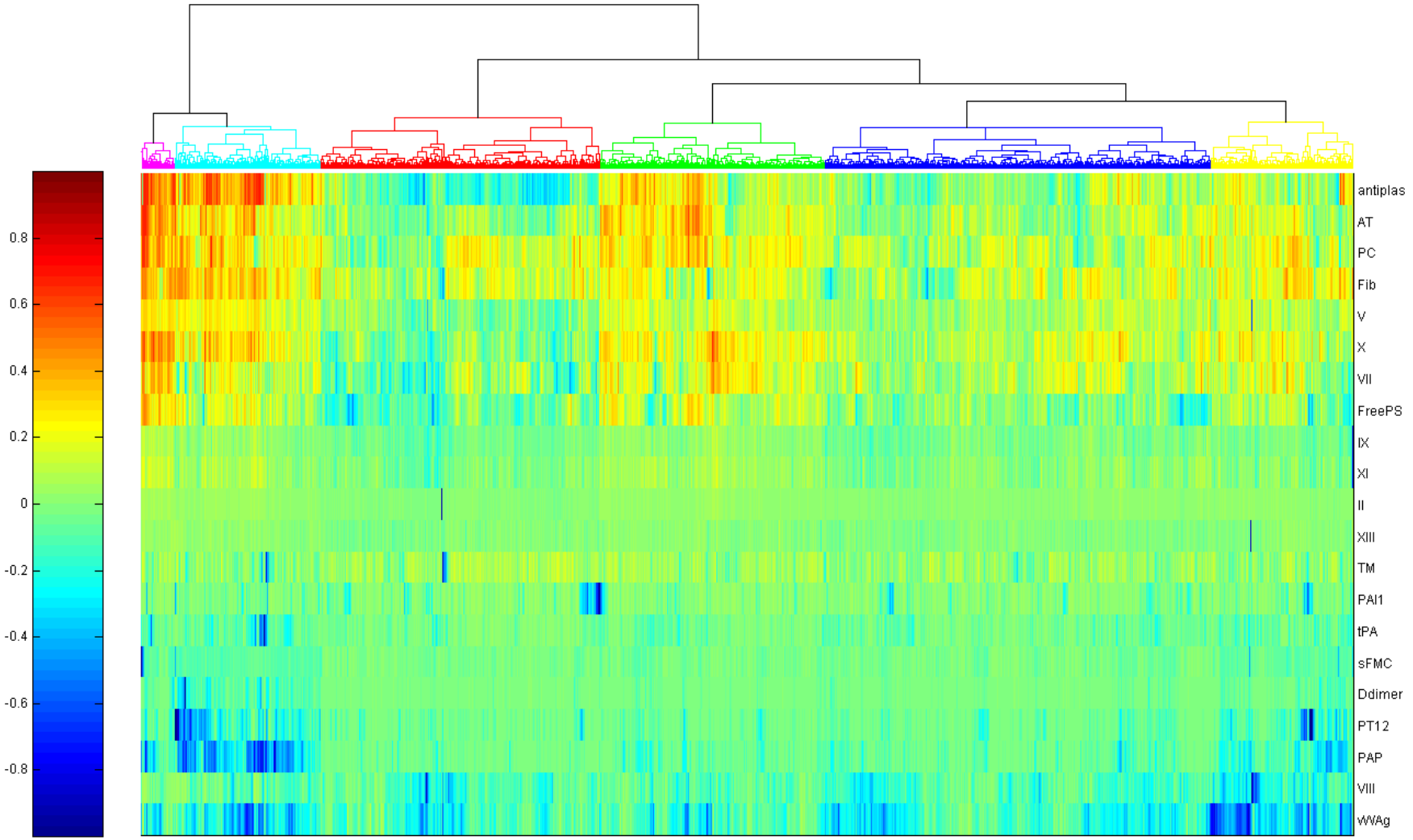

0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
antiplas
AT
PC
Fib
V
X
VII
FreePS
IX
XI
II
XIII
TM
PAI1
tPA
sFMC
Ddimer
PT12
PAP
VIII
vWAg

Figure 6.1 – Preceding page and attached separately

Heat map and dendrogram for biological dataset of 1229 trauma patients. Each column represents a patient and each row represents a variable (annotated on the right of the heat map; antiplas-α2Antiplasmin, AT-Antithrombin, PC- Protein C, Fib- Fibrinogen, V- Factor V, X- Factor X, VII- Factor VII, FreePS- Free Protein S, IX- Factor IX, II- Factor II/Prothrombin, XIII- Factor XIII, TM- Thrombomodulin, PAI1- Plasminogen activator Inhibitor-1, tPA - Tissue type Plasminogen activator, sFMC- Soluble Fibrin monomer complexes, Ddimer- D-Dimer, PT12- Prothrombin fragment 1&2 ,PAP- Plasmin-Antiplasmin complex ,VII-Factor VII ,VWF:Ag- von Willebrand Antigen).

6 clusters are seen as depicted by the different colours on the dendrogram.

Cluster 1 – Dark Blue – 391 patients

Cluster 2 – Green - 227 patients

Cluster 3 – Red - 284 patients

Cluster 4 – Yellow - 145 patients

Cluster 5 – Light Blue - 148 patients

Cluster 6 – Magenta - 34 patients

The colours in each cell represent the value of the normalised variable for that subject. The normalised values are opposite to the true values in relation to the mean of the sample's normal range. Therefore, when considering the original raw variable, red cells depict low levels compared to the normal range and blue depict high levels, with green ('0') being the mean of the normal range for that variable.

6.3.2 Demographics and injury (D&I) dataset

All the D&I data are summarised in tables 6-1 and 6-2. All six clusters show subtle as well as obvious differences in their presenting characteristics. It should be re-iterated here that all these clusters were formed in an unbiased manner from the information in the biological dataset only.

Cluster 3 (C3) is the least injured with a median ISS of 9. This might suggest it was the most "normal" cluster. Clusters 4, 5 and 6 had severe injuries with ISS of >25 (fig 6.3). In terms of specific injury pattern, cluster 5 (C5) showed more polytrauma with injuries in all regions but certainly a predominant element is head injuries compared to other clusters. C6 also present with polytrauma but without the significant head injury element seen in C5. C4 also showed polytrauma but not to the degree of C5 and C6.

It is interesting to note that by clustering the protein data a group has emerged that is older than the other groups (C5). Whether this has been selected by the protein data or whether these subjects are more likely to suffer trauma and then result in particular patterns of coagulation derangement is an interesting question. This group also have fewer males but overall the proportion of males is still high (as expected in the trauma population). C5 also has appreciably lower penetrating injuries with almost 100% being blunt trauma.

C4 and 5 also took the longest to arrive at the hospital post injury (C6 also but did not reach significance). The difference is not is not more than 10-15 minutes but does likely represent the complicated nature of injuries as well as transfer.

The degree of physiological derangement is also important. C2, although not severely injured (as characterised by ISS) were nevertheless shocked overall as a group with high lactate and base deficit. But C4, 5 and 6 were significantly more shocked on arrival. C6 being the most extreme as a group. C6 also received the most fluid resuscitation and blood

product before blood draw. The Haematocrit is also lower for C5 and 6 (although still in the normal range). This would suggest either dilution of perhaps blood loss in these groups.

It is also important to note the temperature on arrival in C5 was significantly lower (fig 6.2), although still not low enough to be labelled hypothermic or in fact at the level where it should affect coagulation(20).

The degree of platelet reduction is also important to note (Fig 6.4). This may simply be due to dilution (Fig 6.4) or some element of “depletion”. Nevertheless it remains above 150,000 per µL and certainly this is not so low to be considered a frank “consumption” such as disseminated intravascular coagulopathy (DIC).

Table 6-1 Demographics and injury characteristics

Significance testing between clusters. * denotes significant difference compared to cluster 1, + denotes significant difference compared to cluster 2 and ‡ denotes significance compared to cluster 3. Demographics and shown as mean (and confidence intervals). Injury characteristics are shown as median (and interquartile range). Significance denotes p<0.05 but in many cases p<0.01

Clusters	1	2	3	4	5	6
Age (years)	41.2(40-43)	43.6(41-46)	38.8(37-41)+‡	39.6(36-43)	48.6(45-52) *	42.5(36-49)
% Male	84(81-88)+	72(66-78) *	81(76-85)	79(72-85)	68(60-75) *‡	62(45-79) *
% penetrating trauma	19(15-23)	17(12-22)	16(12-21)	14(8-19)	3(0-5) *+‡	21(6-35)
% intubated	29(24-33)	33(27-39)	29(23-34)	50(41-58) *+‡	74(67-81) *+‡	62(45-79) *+‡
Time from Injury to ED (mins)	65(62-69)	63(59-67)	69(65-72)	76(72-81) *+‡	79(75-84) *+‡	79(63-95)
Injury Characteristics						
ISS	13(5-22) ‡	13(6-25) ‡	9(4-16) *+	25(15-30) *+‡	30(25-41) *+‡	29(20-40) *+‡
AIS Abdomen & Pelvis	0(0-0)	0(0-0)	0(0-0)	0(0-2) *+‡	0(0-2) *+‡	2(0-3) *+‡
AIS Face	0(0-0)	0(0-0)	0(0-0)	0(0-0)	0(0-1) *+‡	0(0-0)
AIS Thorax	0(0-3) ‡	0(0-3) *‡	0(0-2)	3(0-4) *+‡	3(0-4) *+‡	3(0.5-4) *+‡
AIS Head & Neck	0(0-2)	0(0-3)	0(0-2)	0.5(0-4) *	3(0-5) *+‡	0(0-3.75)
AIS External	0(0-0)	0(0-1)	0(0-0)	0(0-0)	0(0-0) *+	0(0-0)
AIS Extremity & Pelvis	0(0-2)	0(0-3)	0(0-2)	2(0-3) *+‡	2(0-3) *+‡	2(0-3.75) ‡

Table 6-2 Admission physiology and early infusions

Significance testing between clusters. * denotes significant difference compared to cluster 1, + denotes significant difference compared to cluster 2 and ‡ denotes significance compared to cluster 3. Demographics and shown as mean (and confidence intervals). Significance denotes p<0.05 but in many cases p<0.01

Clusters	1	2	3	4	5	6
Admission Physiology						
Glasgow coma score	13(12-13)	12(12-13)	13(12-13)	11(11-12) ***+‡**	9(8-10) ***+‡**	10(7-13)
Systolic blood pressure	135(132-137) **+**	123(119-127) ***‡**	138(135-141) **+**	124(119-129) ***‡**	119(113-125) ***‡**	100(90-109) ***+‡**
Temperature on arrival	36(35.9-36.1)	36(35.8-36.2)	36(35.9-36.2)	35.8(35.6-36)	35.2(34.7-35.7) ***+‡**	35.8(35.2-36.4)
Lactate	2.9(2.6-3.2)	3.1(2.7-3.6)	2.4(2.1-2.6)	3.5(2.9-4) ***+‡**	4.7(4-5.4) ***+‡**	6.8(4.8-8.9) ***+‡**
Base deficit	-1.5(-1.9--1.2) **+**	-2.9(-3.6--2.2) ***‡**	-1.1(-1.5--0.7) **+**	-3.4(-4.2--2.6) ***‡**	-7.6(-8.7--6.4) ***+‡**	-10.1(-13.5--6.7) ***+‡**
Haematocrit	0.41(0.41-0.42) **+**	0.39(0.38-0.4) ***‡**	0.41(0.4-0.42) **+**	0.4(0.39-0.41) *****	0.37(0.36-0.38) ***‡**	0.33(0.25-0.42) ***‡**
Platelet count	238(231-244) **+**	220(212-228) ***‡**	243(235-250) **+**	232(222-242)	194(183-206) ***+‡**	180(154-206) ***‡**
Infusions prior to blood draw						
Crystalloid (mls)	190(154-227) **+**	316(250-383) ***‡**	164(127-201) **+**	281(215-347) ***‡**	423(325-520) ***‡**	982(644-1320) ***‡**
Colloid (mls)	0(0-0)	4(-1.6-9.6)	0(0-0)	0(0-0)	12.2(-5-29.5)	17.2(-18.1-52.6)
Hypertonic saline (mls)	12.3(5.7-18.9)	16.3(5.1-27.6)	5.8(0.6-11.1)	10.1(0.2-19.9)	72(48.8-95.2) ***+‡**	60.3(5.5-115.2) ***‡**
Packed red cells (units)	0(0-0.1)	0.2(0.1-0.3) **‡**	0(0-0) **+**	0.2(0.1-0.3) ***‡**	0.6(0.4-0.9) ***+‡**	0.7(0.1-1.2) ***+‡**

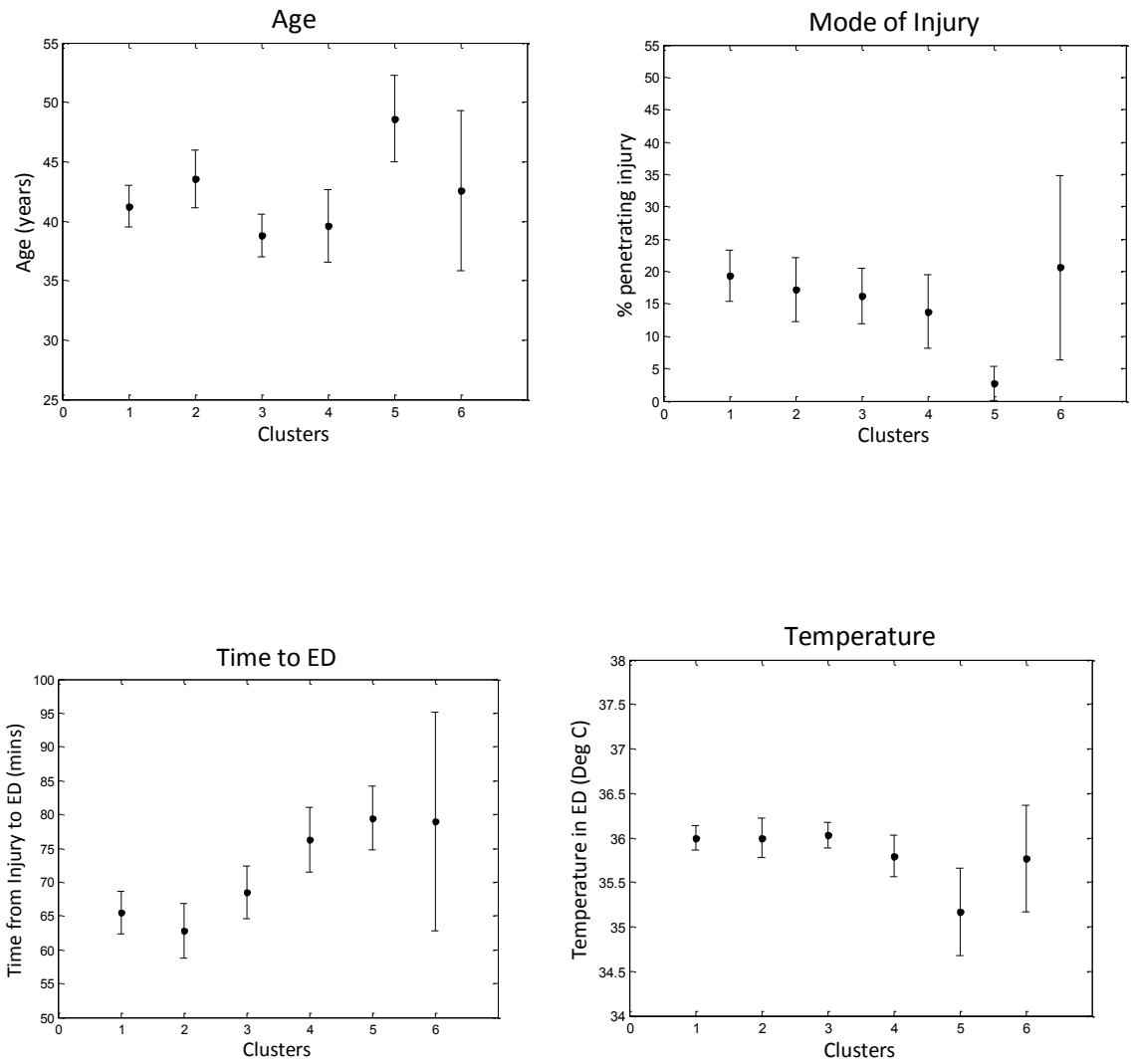
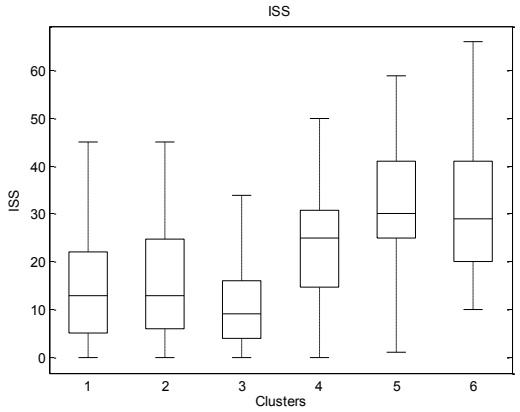
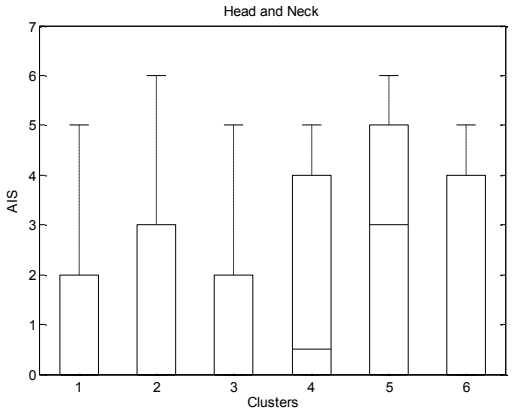


Figure 6.2 Admission demographics

All points are mean and confidence intervals. Significance data is given in the corresponding data tables





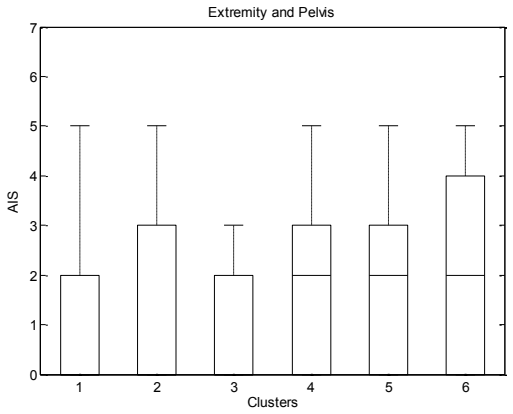


Figure 6.3 Injury Characteristics

Box plots are median and interquartile range for each box and whiskers are 2 standard deviations. Significance data is given in the corresponding data tables

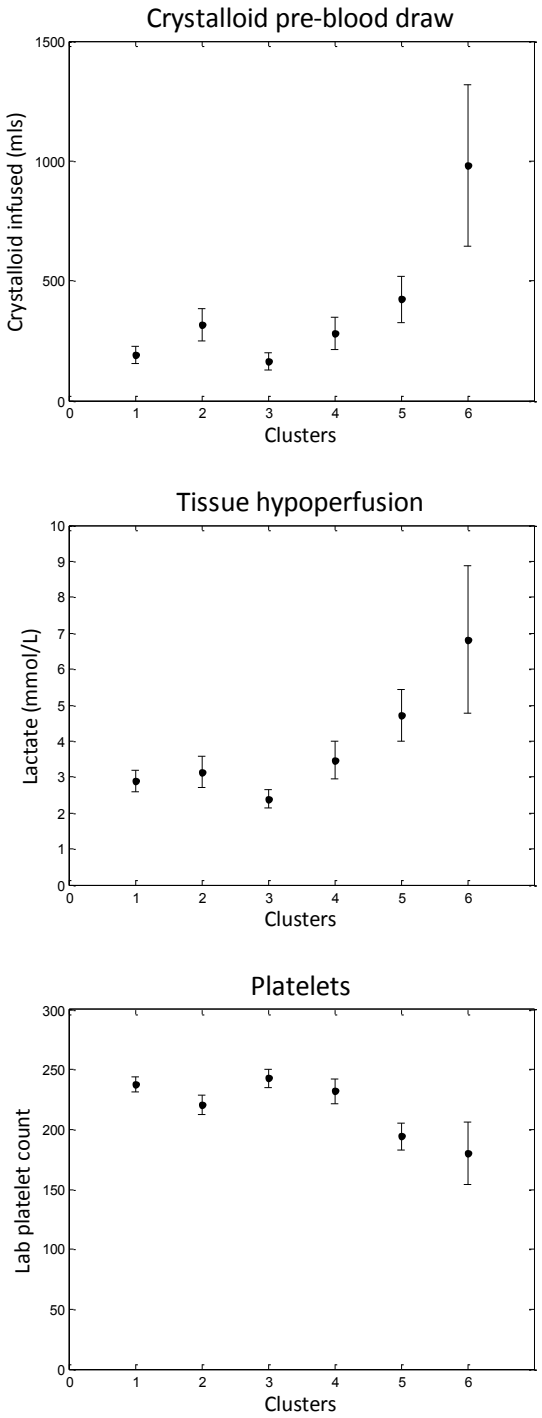


Figure 6.4 Admission Physiology

All points are mean and confidence intervals. Significance data is given in the corresponding data tables

6.3.3 Biological

All the Biological data are summarised in tables 6-3 and 6-4. Significant and interesting derangements are seen in this dataset. This data should also be reviewed with the heat map to hand (figure 6.1) as these patterns can be seen visually on the heat map also.

C1 and C3 appear to have the fewest coagulation derangements. They both have normal INR and fibrinogen levels are maintained. The numbered coagulation factor levels are also normal in both albeit with C1 having slightly lower levels. What is interesting to note however is that C3 has 25% higher PAI-1 than C1. This seems to translate to a lower level of fibrinolytic activation (FA) as evidenced by lower PAP. The PAP is not in the normal range in any group but the lowest is in C3. D-dimers are also lower suggesting less activation and subsequent fibrinolysis.

Looking at groups C2 and C4 we can appreciate a coagulopathy with both arriving in hospital with an INR >1.2 (figure 6.5). C2 in particular is interesting in that these patients seem to have a low injury load (figure 6.3), low levels of shock as evidence by low lactate and base deficit. Nevertheless they still appear coagulopathic. Certainly they show signs of moderate FA with PAP around 3900 mcg/L (figure 6.5) and raised D-dimers. There is a significant reduction in α2-antiplasmin which also suggests significant FA. C4 has similar characteristics but the injury load and level of shock is higher. The coagulopathy here is easier to understand. There are system derangements in keeping with FA of a quite significant level with PAP near 10,000 (figure 6.5), relatively high tPA, significant activation of coagulation as evidenced by raised thrombin production and utilisation of fibrinogen (figure 6.5). There is relative preservation of the numbered coagulation factors (figure 6.6). Overall this cluster shows a fibrinolytic phenotype also.

C5 is a group that demonstrates frank coagulopathy and significant derangements. All are in keeping with fibrinolytic activation as C4 but significantly more pronounced. This cluster

is perhaps the furthest along the continuum of fibrinolysis than the other groups with a mean PAP of 19,500 mcg/L and significant coagulopathy by INR of 1.8. This group is also coagulopathic by APTT suggesting a problem with the factors involved in the traditional intrinsic pathway. In fact if we examine these factors they are becoming globally reduced. Almost all are at their lower limits of normal but are significantly reduced in comparison to C1 and C3 and many also compared to C2.

This trend is taken to the extreme with C6. This is a small group (34 patients) and is characterised by the most severe of coagulopathy in comparison to the other groups with an INR of 3.0 and an APTT ratio of 2.2. The main coagulation system derangement is the global reduction in numbered clotting factors. In some cases as low as 30-40% of normal. In fact FV in this group has 20% of normal activity. The most interesting aspect is that this group is not characterised by severe fibrinolytic activation like C5. There is FA here but PAP and D-dimer levels are around a half of group C5. In addition there seems to be reduced thrombin generation as evidenced by reduced PT1+2. C6 also has the highest fibrinogen utilisation with a mean fibrinogen of only 0.97 g/dL (figure 6.5). Another important finding here is that soluble fibrin monomer complexes (sFMC) are being produced. Infact the level is higher than any other group. This suggests the coagulopathy may be consumptive. Platelets in this group were also significantly reduced in comparison to other groups but were not low enough to meet criteria for DIC. In fact they were still within what would be a normal range for platelets.

Table 6-3 Coagulation factors 1

Significance testing between clusters. * denotes significant difference compared to cluster 1, + denotes significant difference compared to cluster 2 and ‡ denotes significance compared to cluster 3. Protein data is shown as mean (and confidence intervals). Significance denotes p<0.05 but in many cases p<0.01

Clusters	1	2	3	4	5	6
PAI-1	31.1(28.4-33.7)‡	26.9(23.3-30.5) ‡	41.6(35.1-48.1) *+	39.6(33.1-46.0) +	35.5(30.9-40.2)	25.4(14.8-36.0)
PAP	3619(3312-3925) ‡	3903(3494-4311) ‡	2186(1963-2409) *+	9615(8610-10621) *+‡	19498(18078-20918) *+‡	11368(8179-14557) *+‡
tPA	15.2(14.3-16.0) ‡	13.6(12.4-14.7)	11.5(10.5-12.5) *	19.1(17.1-21.0) *+‡	26.3(22.5-30.1) *+‡	22.6(16.0-29.1) *+‡
PT12	1356(1228-1484)	1282(1113-1450)	1171(1021-1320)	4272(3531-5013) *+‡	6338(5561-7115) *+‡	2394(1677-3110)
D-Dimer	16,291(14,054-18,528) ‡	15,984(12,963-19,005) ‡	9,595(7,806-11,384) *+	50,062(42,150-57,974) *+‡	111,333(94,608-128,058) *+‡	59,485(38,133-80,838) *+‡
Fibrinogen	2.37(2.30-2.44) +	2.02(1.94-2.11) *+‡	2.36(2.29-2.43)	1.72(1.63-1.82) *+‡	1.22(1.12-1.31) *+‡	0.97(0.76-1.19) *+‡
sFMC	89(80-97)	98(85-111)	72(63-81)	229(203-255) *+‡	249(234-265) *+‡	353(170-537) *+‡
α2-Antiplasmin	99(98-101) +‡	76(73-79) *‡	122(119-125) *+	88(84-92) *+‡	45(41-48) *+‡	31(24-39) *+‡
Prothrombin time	11.5(11.4-11.7)	13.8(11.8-15.9)	11.0(11.0-11.2)	14.4(11.2-17.6)	19.5(15.1-24.0) *+‡	32.6(16.2-49.0) *+‡
INR	1.1(1.1-1.1)	1.3(1.1-1.5)	1.1(1.0-1.1)	1.4(1.1-1.6)	1.8(1.4-2.2) *+‡	3.0(1.6-4.5) *+‡
APTT ratio	0.9(0.9-0.9)	1.0(1.0-1.1)	0.9(0.9-0.9)	0.9(0.8-1.0)	1.4(1.3-1.6) *+‡	2.2(1.6-2.9) *+‡

Table 6-4 Coagulation factors 2

Significance testing between clusters. * denotes significant difference compared to cluster 1, + denotes significant difference compared to cluster 2 and ‡ denotes significance compared to cluster 3. Protein data is shown as mean (and confidence intervals). Significance denotes p<0.05 but in many cases p<0.01

Clusters	1	2	3	4	5	6
II	93(92-95) +‡	74(72-76) *‡	108(100-115) *+	83(81-86) *+‡	66(63-69) *+‡	38(32-43) *+‡
V	85(83-87) +‡	66(63-69) *‡	104(100-107) *+	74(69-78) *‡	41(37-45) *+‡	20(15-25) *+‡
VIII	265(254-277) +	206(194-219) *‡	263(246-280) +	372(343-401) *+‡	206(186-225) *‡	121(92-151) *+‡
VII	90(88-92) +‡	74(71-77) *‡	112(109-115) *+	87(83-91) +‡	83(79-87) *+‡	44(38-50) *+‡
XI	105(102-107) +‡	77(74-80) *‡	115(112-119) *+	110(103-118) +‡	78(73-83) *‡	38(31-45) *+‡
IX	115(112-118) +‡	84(81-87) *‡	125(122-129) *+	120(111-128) +‡	83(78-88) *‡	45(37-52) *+‡
X	90(89-92) +‡	71(69-74) *‡	109(107-112) *+	82(79-85) *+‡	67(64-70) *+‡	37(31-42) *+‡
XIII	115(112-118) +	99(95-103) *	116(112-120) +	107(95-120) *‡	87(82-91) *+‡	66(58-75) *+‡
Von Willebrand Antigen	224(217-232) +‡	192(181-203) *	195(186-204) *	348(329-367) *+‡	293(277-309) *+‡	217(179-255) ‡
Antithrombin	94(93-95) +‡	70(68-73) *‡	100(98-101) *+	84(82-86) *+‡	68(66-71) *‡	39(34-45) *+‡
Protein C	102(101-104) +	77(75-79) *‡	102(99-105) +	91(87-95) *+‡	75(72-78) *‡	48(41-56) *+‡
Free Protein S	104(102-106) +‡	79(76-82) *‡	112(110-115) *+	94(90-99) *+‡	77(73-81) *‡	42(36-48) *+‡

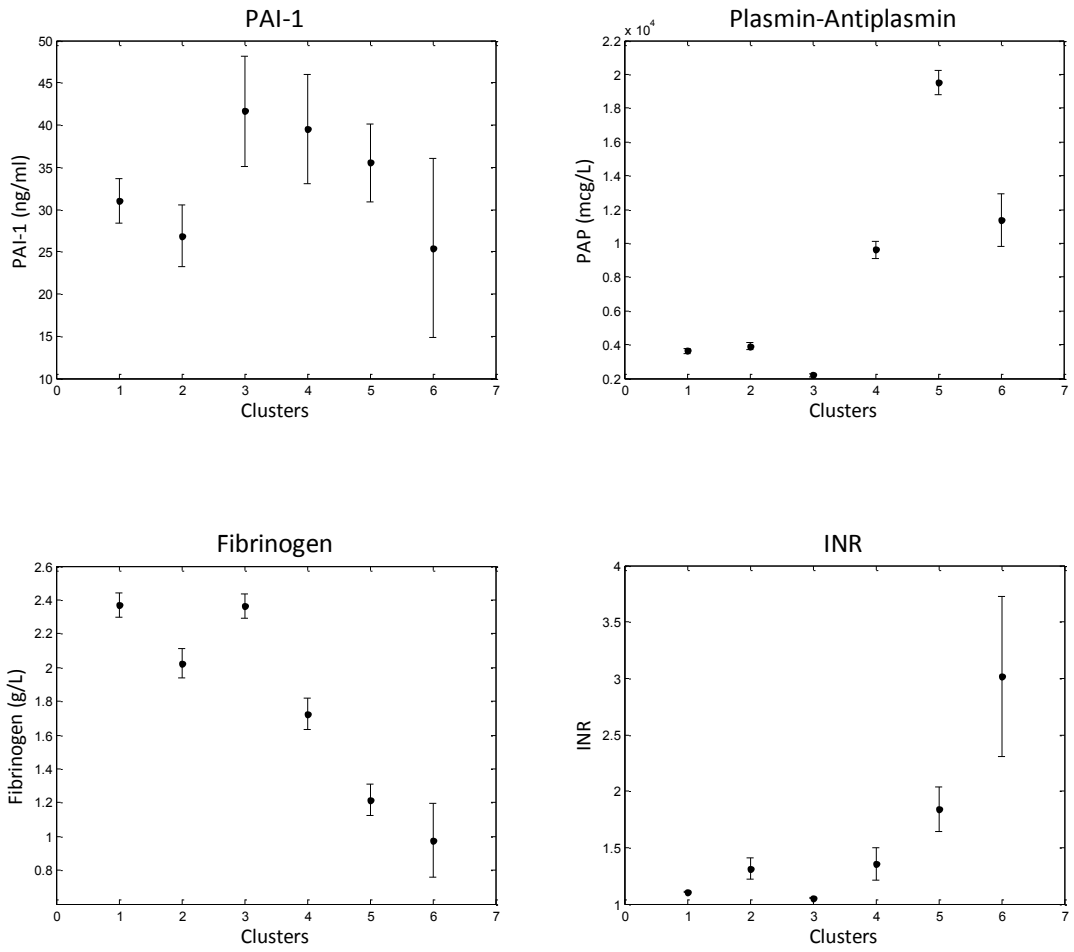


Figure 6.5 Coagulation factors 1

All points are mean and confidence intervals. Significance data is given in the corresponding data tables

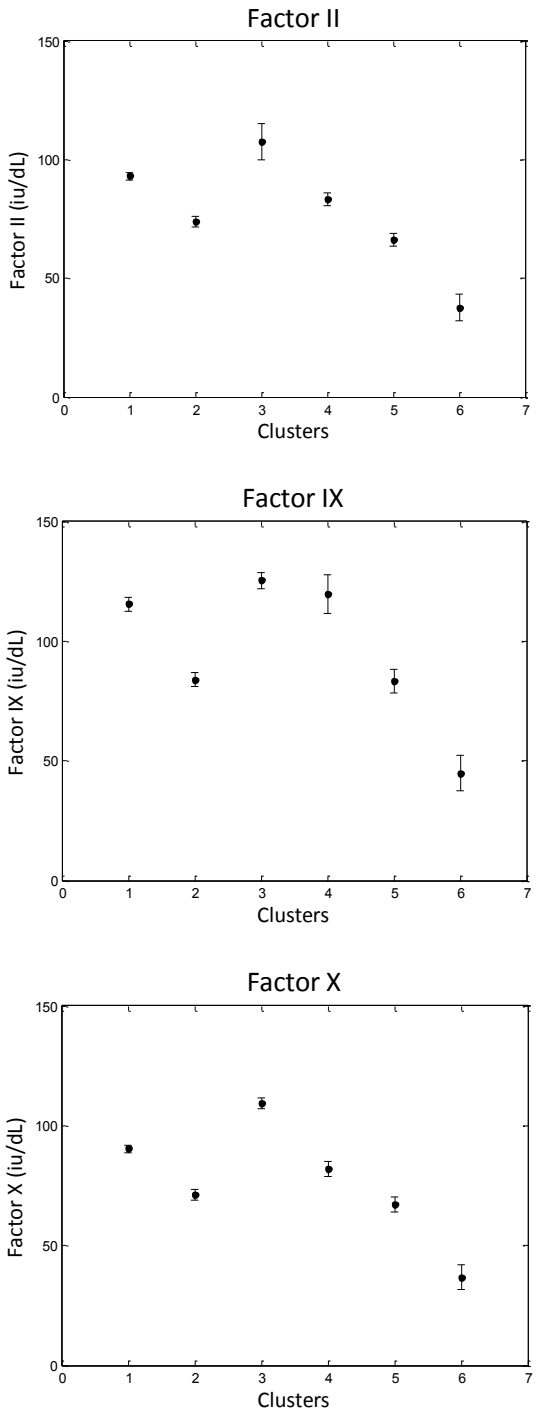


Figure 6.6 Coagulation factors 2

All points are mean and confidence intervals. Significance data is given in the corresponding data tables

6.3.4 ROTEM

The ROTEM data is summarised in table 6-5. The degree of derangement seen on ROTEM in some ways simply reflect the derangements see in the above 2 sections.

There was a prolongation of the clotting time but only in the two most severely coagulopathic groups. And even though these groups were obviously coagulopathic by INR they were not definitely so by ROTEM based on our groups previous exploration of CA5(37). C5 was on the threshold of coagulopathy by ROTEM. C6 was coagulopathic by ROTEM but the variability in this group was much higher than others (figure 6.7). Over all clot strength (MCF) was reduced compared to the 'normal' groups.

The ExTEM maximum lysis in the two extreme groups was significantly raised. This would be expected given the biological data findings. It is interesting though that aporotinin was able to reverse the lysis over all such that the ApTEM maximum lysis no longer showed a significant lysis (figure 6.8)

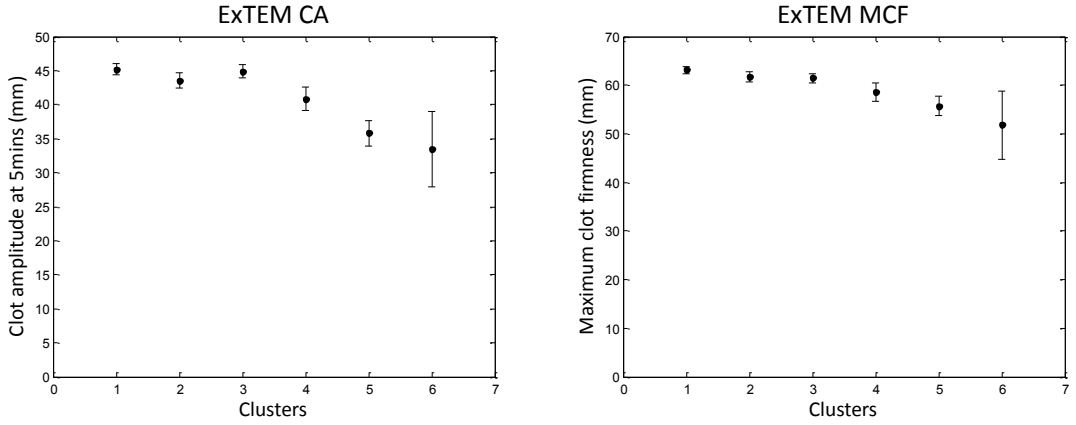


Figure 6.7 ExTEM parameters

All points are mean and confidence intervals. Significance data is given in the corresponding data tables

Table 6-5 ROTEM measures of Coagulopathy

Significance testing between clusters. * denotes significant difference compared to cluster 1, + denotes significant difference compared to cluster 2 and ‡ denotes significance compared to cluster 3. ROTEM data is shown as mean (and confidence intervals). Significance denotes p<0.05 but in many cases p<0.01

Clusters	1	2	3	4	5	6
ExTEM						
Clotting time	59(57-61)	57(54-59)	63(60-67)	65(60-69)	116(65-167) *+‡	132(69-194) *+‡
Clot Amplitude 5 min	45(44-46)	44(42-45)	45(44-46)	41(39-43) *‡	36(34-38) *+‡	34(28-39) *+‡
Alpha-angle (degrees)	72(71-73)	71(70-72)	72(71-73)	69(67-70) *‡	65(64-67) *+‡	65(59-71) *+‡
Maximum clot firmness	63(62-64)	62(61-63)	61(60-62)	59(57-60) *+‡	56(54-58) *+‡	52(45-59) *+‡
% Maximum lysis	8(7-9)	8(7-9)	8(7-8)	7(6-8)	19(13-25) *+‡	22(4-39) *+‡
FibTEM						
Clot amplitude 5 min	13(13-14)	13(12-13)	13(13-14)	10(10-11) *+‡	9(8-9) *+‡	8(7-10) *+‡
Maximum clot firmness	16(15-17)	16(14-17)	16(15-17)	13(12-14) *+‡	11(10-12) *+‡	11(9-13) *+‡
ApTEM						
Clot amplitude 5 min	45(44-46)	43(41-44)	45(44-46)	40(38-42) *‡	35(34-37) *+‡	31(26-37) *+‡
Maximum clot firmness	62(61-63)	61(60-62)	61(60-62)	58(56-60) *+‡	55(53-58) *+‡	51(45-58) *+‡
% Maximum lysis	7(6-9)	7(6-8)	8(7-9)	7(6-9)	6(4-9)	11(0-22)

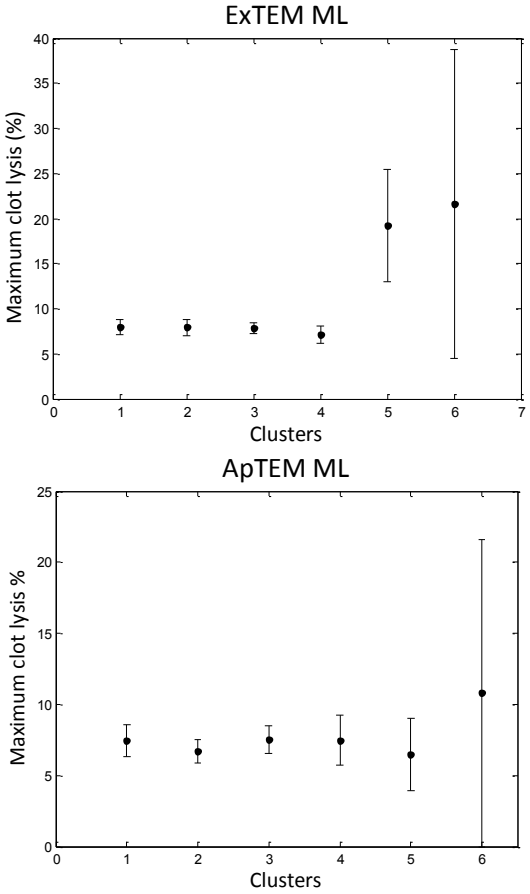


Figure 6.8 Lysis seen on ROTEM

All points are mean and confidence intervals. Significance data is given in the corresponding data tables

6.3.5 Resuscitation fluids (RF)

The fluids received in the first 24 hours are summarised in table 6-6 below. C3 received the lowest amount of RF over all further suggesting this is the most uninjured or normal group. C2 which had low injury load and not particularly deranged physiology still received a significant amount of blood and blood products (figure 6.9).

C5 received the highest mean volumes of hypertonic saline. This also reinforces our earlier observations that this group had a significant number of head injuries.

C6 was the cluster utilising the highest number of blood and blood products. If this group does indeed have a consumptive coagulopathy, this strategy may not have been conducive to resuscitation. This underlines the importance of early diagnosis and of this type of work.

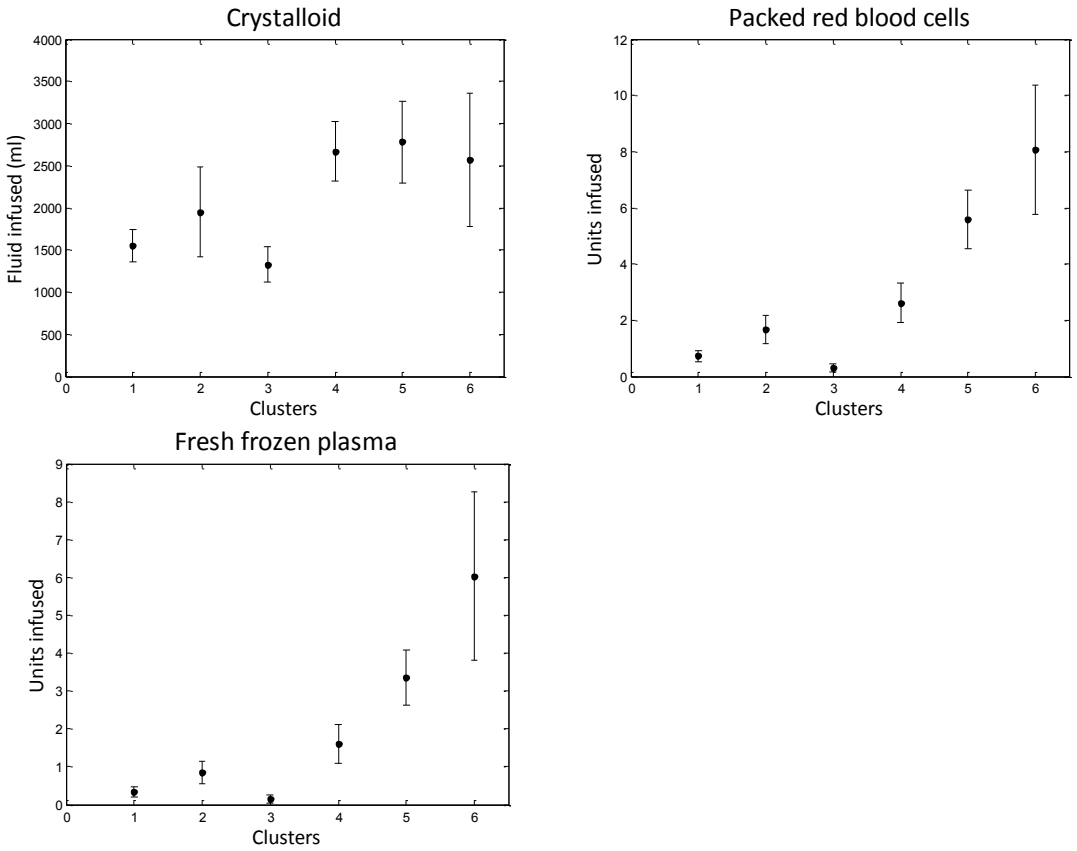


Figure 6.9 Ongoing Fluid Requirements

Table 6-6 Fluid Resuscitation

Significance testing between clusters. * denotes significant difference compared to cluster 1, + denotes significant difference compared to cluster 2 and ‡ denotes significance compared to cluster 3. Fluid data is shown as mean (and confidence intervals). Significance denotes p<0.05 but in many cases p<0.01

	1	2	3	4	5	6
Crystalloid (mls)	1548(1359-1737)	1951(1417-2486)	1328(1119-1537)	2669(2318-3019) *+‡	2781(2298-3265) *+‡	2569(1776-3361) ‡
Colloid (mls)	254(190-319)	231(155-308)	163(103-223)	700(525-876) *+‡	798(607-988) *+‡	473(226-720) ‡
Packed red cells	0.72(0.53-0.91)	1.68(1.18-2.18) ‡	0.3(0.16-0.44) +	2.62(1.92-3.32) *+‡	5.58(4.54-6.62) *+‡	8.06(5.77-10.35) *+‡
Fresh frozen plasma	0.34(0.21-0.46)	0.85(0.55-1.15) ‡	0.15(0.05-0.26) +	1.6(1.08-2.12) *+‡	3.35(2.63-4.07) *+‡	6.03(3.81-8.25) *+‡
Platelets	0.08(0.05-0.12)	0.27(0.16-0.38) ‡	0.03(0-0.06) +	0.29(0.17-0.41) *‡	1(0.73-1.27) *+‡	1.35(0.67-2.04) *+‡
Cryoprecipitate	0.03(0.01-0.06)	0.13(0.05-0.2)	0.01(0-0.03)	0.32(0.16-0.48) *+‡	0.63(0.43-0.82) *+‡	0.41(0.03-0.8) *‡
Hypertonic saline (mls)	4(-1-10)	13(3-23)	2(-1-5)	9(0-18)	25(9-41) *‡	0(0-0)

6.3.6 Outcomes

Outcomes for all groups are shown in table 6-7 below. The outcomes for C1-3 were overall better than the other groups as one might expect. Groups C4-6 had gradually worse outcomes respectively over all. What is surprising however, is the relatively low mortality rate in C6. Given the extremes of coagulation derangement one might expect the mortality to be much higher than it was. The fact that the highest mortality was in C5 (and not C6) (figure 6.10) may again reflect the degree of head injuries seen in that group.

C4-6 also had worse critical care, vasopressor and ventilator days suggesting overall more organ and possibly multi-organ dysfunction.

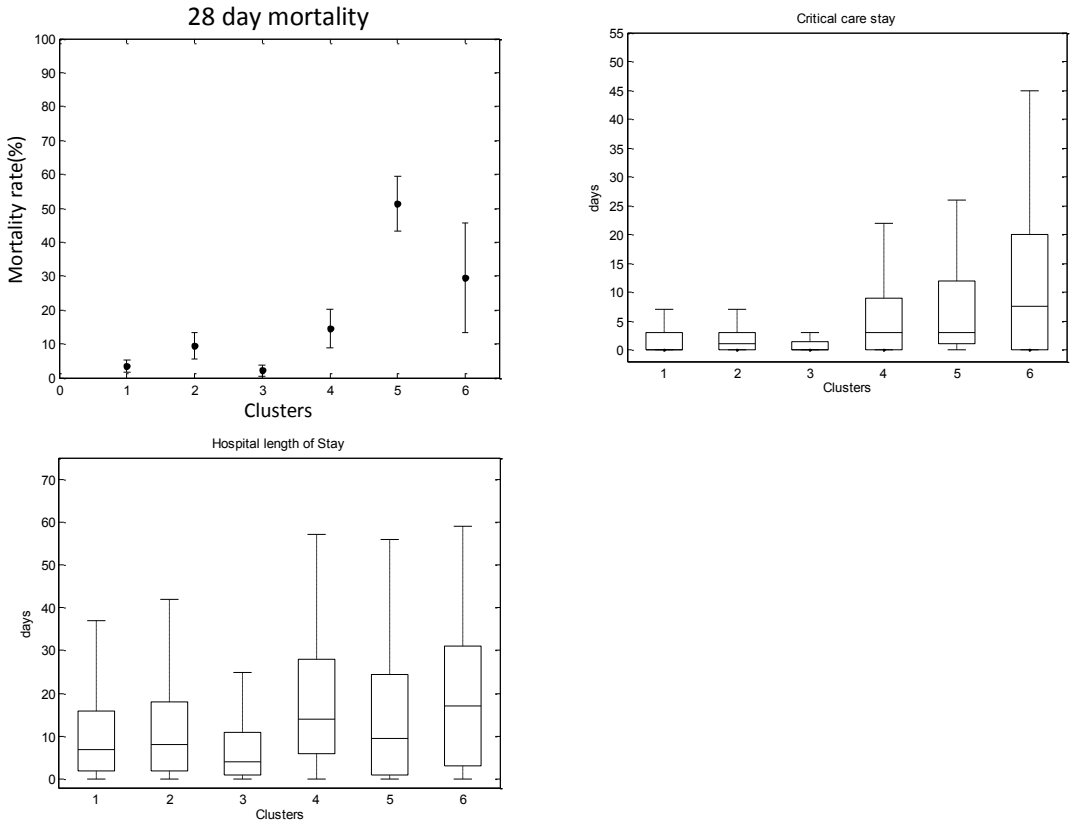


Figure 6.10 Outcomes

Table 6-7 Outcome measures

Significance testing between clusters. * denotes significant difference compared to cluster 1, + denotes significant difference compared to cluster 2 and ‡ denotes significance compared to cluster 3. Mortality is shown and mean and confidence intervals. All other outcome data is given at median and interquartile range. Significance denotes p<0.05 but in many cases p<0.01

Clusters	1	2	3	4	5	6
Mortality %	3(2-5)	9(6-13)	2(0-4)	14(9-20) ***‡**	51(43-59) ***+‡**	29(13-46) ***+‡**
Critical care days	3(3-4) **‡**	4(3-5) **‡**	2(1-2) ***+**	6(5-8) ***+‡**	7(6-9) ***+‡**	12(7-16) ***+‡**
Vasopressor days	1(1-1)	1(0-1)	0(0-1)	2(1-2) ***+‡**	2(2-3) ***+‡**	4(0-7) **‡**
Renal replacement therapy	0(0-0)	0(0-0)	0(0-0)	0(0-0)	0(0-1) ***‡**	0(0-0)
Ventilator days	2(1-2)	2(1-3) **‡**	1(0-1) **+**	4(3-6) ***+‡**	5(4-6) ***+‡**	6(3-10) ***+‡**
Length of stay	14(11-17) **‡**	16(12-20) **‡**	12(7-18) ***+**	22(18-26) ***+‡**	16(13-19) **‡**	25(11-39) **‡**

6.4 discussion

Understanding and interpreting the coagulation system derangements in trauma continues to be a challenge. But with more robust data collection and with analysis techniques borrowed from other disciplines progress can be made.

With this work we have primarily shown that it is possible to use a hierarchical clustering on a medium sized protein dataset to classify derangements in an unbiased way. We have shown that the results obtained are meaningful and do seem to correlate with previous results on this important disease process. We have shown this can be done in an unbiased way and has therefore potential to discover unexpected groups (such as C6).

Other teams have used clustering in trauma coagulation research. The Pittsburgh group have used it on an animal model with relatively small numbers(86). White and colleagues have applied it to their human dataset(87). This has shown meaningful results but again those are relatively small numbers. The utility of clustering small groups is not immediately obvious. To our knowledge this current work represents the largest human dataset in trauma for this type of analysis to date.

This method has been useful in understanding the patterns of derangement without a priori assumptions or bias. Hierarchical clustering has identified 6 different groups. One of these (C3), is likely to represent minimally injured patients with very few (if any) physiological sequelae. Even in this group, levels of coagulation proteins were abnormal in comparison to their reference ranges. PAP, tPA, PT1+2 and D-dimer were raised, suggesting a level of activation of the coagulation system.

The remaining clusters are all at different levels of activation, likely all of the fibrinolytic system as we see PAP and D-dimer going higher as we go from C1 to C2, C4, and C5. C5 is the group with the most severe form of fibrinolysis. The difference between this and other

clusters is mainly the presence of the significant head injuries in this group. It has long been thought that head injuries are a contributing factor to development of hyperfibrinolysis. Certainly there is a strong association between coagulopathy and head injury(88). Our data support this conclusion. But they possibly go further. We have shown how severe a derangement there actually is. Along with the extreme rise in PAP and D-dimer there is also a relative decrease in the numbered clotting factors. All this together may contribute to the significant functional coagulopathy in these patients. The other striking feature is how quickly this coagulopathy develops. Cluster C5 had a scene to hospital time of about 1 hour and 15 minutes. Part of the CRASH-2 trial findings was to give TXA as quickly as possible after injury. The aim of the CRASH-3 trial is similar but the cut-off to give the drug is 8 hours. With such a significant coagulopathy developing so quickly it simply underlines the need for early intervention. It also adds weight to the argument that TXA should be given on much earlier timescales than this to avoid secondary bleeds and exacerbation of brain injury.

A further important finding is that of Cluster 6. This is a small part of the patient cohort. This group has only 34 patients out of 1229 analysed, i.e. 2.76%. Nevertheless it is an important group because it displays a separate mechanism of coagulopathy. This severe coagulopathy appears to have signs of a consumption and not pure fibrinolysis alone. The finding of the high SFMC is key to this discussion. Soluble Fibrin Monomer complexes are a part of the diagnostic criteria to diagnose DIC as defined by the International Society of Thrombosis and Haemostasis (ISTH)(89,90). However, with platelet levels being maintained this draws into question whether this can truly be DIC or not. Certainly other groups, particularly the Japanese critical care fraternity, have been strong advocates of DIC being the main coagulopathy in trauma for some time(91,92). But our observations show a small group in a much larger cohort that does not display DIC as a whole.

Why these patients should develop a consumptive coagulopathy should also be questioned. Certainly they have a high injury load (ISS-29) but don't have as many head injuries as C5. The rate of penetrating injury is highest in this group also. Meaning, this could be a group with direct vascular injury and massive early blood loss. Certainly they have utilised the most prehospital IV fluids and blood products. Even in the first 24 hours of admission they continue to use blood and products more than the other groups. What is certainly curious is the relatively low mortality in this group (29% vs 51% for C5) given the severity of coagulation derangement; again, suggesting that perhaps acute vascular bleeds that have been surgically dealt may be a feature of this group.

Chapter 7

7.1 Summary of findings

The main focus of this body of work has been to understand the mechanisms behind the acute coagulopathy of trauma. Chapter three has described the incidence of fibrinolytic activation in trauma patients. Although there had been previous studies to understand the fibrinolytic component of coagulopathy, interest was renewed since the publication of the CRASH-2 trial. Our description of the incidence of activation and the magnitude of derangement has gone some way to explaining why a pragmatic approach to treating fibrinolysis in trauma should be successful. In doing so we have described the scale of a problem which had, until now, not been fully appreciated. There are advocates who suggest treatment of fibrinolysis should only be undertaken with viscoelastic test evidence. We have demonstrated that fibrinolytic activation is largely undetectable by thromboelastometry (apart for the most severe cases) and even moderate FA confers a significant risk of mortality and morbidity which cannot be ignored. This coupled with the scale of fibrinolytic activation adds weight to the evidence for use of antifibrinolytics in trauma as an agent in pts with a risk of blood loss.

Explaining the specific mechanisms is more difficult. Chapter 4 has attempted to understand the role of three specific factors which have been implicated in the modulation of fibrinolysis. We have shown that PAI-1 may well have an influence on the level of FA irrespective of the influence of tPA on promoting lysis. This could clearly be a possible locus for molecular intervention.

FXI has been implicated in the curtailing of fibrinolysis. We have shown that at maintained levels this is unlikely the case, but depletion of FXI may well be associated with fibrinolysis. This mechanism has previously been postulated to work through thrombin generation and TAFI. We have however also demonstrated that circulating plasma TAFI does not seem to have any influence on fibrinolysis curtailment. This does not speak of the TAFI that may

exert effect form a platelet source. This work would be much more challenging but may well be required if the mechanism is to be better understood.

The final section is perhaps the most exciting. Of course for its findings, but also due to the potential that it may afford. In this work we have developed a technique commonly used in other fields to analyse large amounts of data which would be otherwise difficult. In doing so we have shown that this is a relevant technique in current analyses. Its findings are to some extent validation of the process and function of the method. Our aim was to examine this dataset in an unbiased manner and we have achieved that. In doing so a possibly important coagulopathy has been described. It may only make up a small percentage of cases but had we used conventional techniques to analyse such a large dataset perhaps we would not have shown this derangement.

The most important clinical or biological finding of this section of work is to show that fibrinolysis is the overriding coagulopathy in trauma. Over the years various mechanisms have been postulated and supported. The 3 main current prevailing theories come from various groups; our group in London (under Professor Brohi), the Japanese group (under Professor Gando) and the Denver group (under EE Moore). The London group has long been an advocate for the primary fibrinolysis mechanism likely mediated by aPC (30). The Japanese group has advocated DIC as a mechanism for many years and more recently this has been re-described as DIC with fibrinolytic phenotype(91). The Denver group have advocated a theory based on fibrinolysis shutdown (93) They have also been cautious in recommending early use of antifibrinolytics without diagnosis beforehand (94).

The findings of this current body of work would support primary fibrinolysis being the predominant mechanism. In fact we have found no evidence that at admission either DIC or fibrinolysis shutdown are relevant mechanisms. Moreover, the assertion by the Denver group that antifibrinolytics should be withheld until a diagnosis of fibrinolysis is

questionable given that we have shown the almost ubiquitous activation of fibrinolysis in all but the least injured patients. In addition as in previous chapters we have shown the early biochemical markers of fibrinolysis with absence of lab or point of care markers. Delaying use of antifibrinolytics in this context could be potentially dangerous.

The theory of fibrinolysis shutdown is largely based on TEG analysis, and to date, is not backed up by laboratory protein data. This may be questionable since we have already shown that ex-vivo viscoelastic tests may not be sensitive at detecting fibrinolysis. One of the strengths of this work is therefore the protein level analysis revealing fibrinolysis and not fibrinolysis shutdown or DIC.

The analysis does show the main derangement of coagulation to be fibrinolysis and shows again how even moderate levels of injury can result in FA, mirroring the findings of chapter 3. To reiterate; hierarchical clustering has not demonstrated any other major coagulopathy.

Therefore we would suggest that fibrinolysis is likely the main and certainly the most clinically important coagulopathy found on admission in major trauma.

7.2 Strengths and weaknesses of this work

To our knowledge, this is the largest non-pragmatic prospective study of trauma coagulation to date. Very large trauma registries exist but have issues retrospectively analysing data. For one, if a clinical question needs to be answered and he material collected is inadequate, retrospective analysis will not work. When data is collected prospectively (as we have done here) we are able to capture much more information that is useful and relevant. But this also creates issues for analysing such large datasets. We therefore need to leverage modern data analysis techniques. Our technique may not be perfect and even primitive in comparison to gene expression and modern mathematical techniques. But we have taken this step with the intention to move forward in this field.

My colleagues Zane Perkins and Simon Glasgow are involved in similar projects. One is using Bayesian network prediction tools to predict outcomes on an individual level and the other; an agent based modelling environment to predict population and public health requirements in major trauma incidents. This is exciting time moving forward and we are privileged to contribute to this. This work has also meant close relationships with computer science and mathematics departments across our university site. These are relationships that our group will continue to benefit from.

The novelty of this work specifically is that we have repurposed old techniques in other sciences to good effect on the largest cohort of trauma subjects recruited to date.

Over the past few years of recruiting patients in an accident an emergency environment using the PLAR consent process we have been very successful indeed. There has been a very low rate of patients who were not able to consent or withdrew consent after the process. This shows that this type of research can be performed in the emergency setting. This has reaped benefits on a local and international level. There is now an ED research team that started recruiting to their own trials at times using our expertise from ACITII. Our encouragement and guidance of their research practise has been instrumental in this process. Our collaboration with European centres has flourished. Fellows from their centres have regularly visited us to take on board our practice in their own countries. Through this collaboration we have increased the recruitment to our own study.

Our constant presence in the ED and our use of ROTEM has meant that ED has now acquired their own ROTEM machine which can be used for any patient. This was a direct result of our familiarity with this research tool and our support in clinical practice.

Data collection from this type of patient group has been challenging at times. Patients in extremis do not lend themselves well to having blood taken continuously or regularly. This

has led to gaps in our data at times. We have worked very hard to reduce this data loss. A result of this can be seen in the final chapters where a significant number of subjects had to be excluded due to missing data. This is almost always a result of deterioration of a very small sample or from continuous processing of the same sample for so many tests. In future it may be possible to gain all this information from minute samples of blood on to protein detection chips. Until then we may continue to suffer from missing data.

It is also important to note that much of the work above and through ACITII is observational work. It results in conclusions that can be at best associations and correlations. This is still an important part of practice but the observations we make and conclusions we derive must be tested in a clinical interventional or in a in vivo non-human setting. There are other members of our group who continue to do this.

One major disappointment was the difficulty with the TM ELISA. Had this ELISA not changed we may have been able to include much of the TM data which would undoubtedly help in understanding these mechanisms further.

7.3 Future work

This current work is a limited view of coagulopathy. It is limited by virtue of being data collected only on the patient's admission. We currently don't have a significant idea of the natural history of coagulopathy over time and how our current interventions affect it. Some work has been carried out on bleeding patients but as yet we don't have full datasets on 24 hour and 72 hour time point samples. If we are able to study those time points also it will surely give us a fuller understanding. This would of course introduce many more variables, including time variables, but our use of clustering would enable those datasets to be analysed in a similar manner.

Bibliography

1. Haagsma JA, Graetz N, Bolliger I, Naghavi M, Higashi H, Mullany EC, et al. The global burden of injury: incidence, mortality, disability-adjusted life years and time trends from the Global Burden of Disease study 2013. Inj Prev . 2015;1–16.

2. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012;380(9859):2095–128.

3. No Title [Internet]. Statistics, Office of National. 2014. Available from: http://www.ons.gov.uk/ons/rel/vsob1/mortality-statistics--deaths-registered-in-england-and-wales--series-dr-/2014/sty-what-do-we-die-from.html

4. Baker CC, Oppenheimer L, Stephens B, Lewis FR, Trunkey DD. Epidemiology of trauma deaths. Am J Surg. 1980 Jul;140(1):144–50.

5. Trunkey DD. Trauma. Accidental and intentional injuries account for more years of life lost in the U.S. than cancer and heart disease. Among the prescribed remedies are improved preventive efforts, speedier surgery and further research. Sci Am. 1983 Aug;249(2):28–35.

6. McGwin G, Nunn AM, Mann JC, Griffin R, Davis GG, MacLennan P a, et al. Reassessment of the tri-modal mortality distribution in the presence of a regional trauma system. J Trauma. 2009;66(2):526–30.

7. Sordi R, Nandra KK, Chiazza F, Johnson FL, Cabrera CP, Torrance HD, et al. Artesunate Protects Against the Organ Injury and Dysfunction Induced by Severe Hemorrhage and Resuscitation. Ann Surg. 2016 Feb 1;XX(X):1.

8. Vu EN, Schlamp RS, Wand RT, Kleine-Deters GA, Vu MP, Tallon JM. Prehospital Use of Tranexamic Acid for Hemorrhagic Shock in Primary and Secondary Air Medical Evacuation. Air Med J. Air Medical Journal Associates; 2013;32(5):289–92.

9. Brown JB, Sperry JL, Fombona A, Billiar TR, Peitzman AB, Guyette FX. Pre-Trauma Center Red Blood Cell Transfusion Is Associated with Improved Early Outcomes in Air Medical Trauma Patients. J Am Coll Surg. American College of Surgeons;

2015;220(5):797–808.

10. Huber-Wagner S, Lefering R, Qvick L-M, Körner M, Kay M V, Pfeifer K-J, et al. Effect of whole-body CT during trauma resuscitation on survival: a retrospective, multicentre study. Lancet. Elsevier Ltd; 2009;373(9673):1455–61.

11. Martinelli T, Thony F, Decléty P, Sengel C, Broux C, Tonetti J, et al. Intra-aortic balloon occlusion to salvage patients with life-threatening hemorrhagic shocks from pelvic fractures. J Trauma. 2010 Apr;68(4):942–8.

12. Roberts DJ, Bobrovitz N, Zygun DA, Ball CG, Kirkpatrick AW, Faris PD, et al. Indications for use of thoracic, abdominal, pelvic, and vascular damage control interventions in trauma patients. J Trauma Acute Care Surg. 2015;79(4):568–79.

13. Cripps MW, Kutcher ME, Daley A, McCreery RC, Greenberg MD, Cachola LM, et al. Cause and timing of death in massively transfused trauma patients. J Trauma Acute Care Surg. 2013;75(2 Suppl 2):S255–62.

14. Demetriades D, Murray J, Charalambides K, Alo K, Velmahos G, Rhee P, et al. Trauma fatalities: Time and location of hospital deaths. J Am Coll Surg. 2004;198(1):20–6.

15. Demetriades D, Kimbrell B, Salim A, Velmahos G, Rhee P, Preston C, et al. Trauma deaths in a mature urban trauma system: is “trimodal” distribution a valid concept? J Am Coll Surg. 2005;201(3):343–8.

16. Pang JM, Civil I, Ng A, Adams D, Koelmeyer T. Is the trimodal pattern of death after trauma a dated concept in the 21st century? Trauma deaths in Auckland 2004. Injury. 2008;39:102–6.

17. Pfeifer R, Tarkin IS, Rocos B, Pape H-C. Patterns of mortality and causes of death in polytrauma patients--has anything changed? Injury. 2009 Sep;40(9):907–11.

18. Sauaia A, Moore FA, Moore EE, Moser KS, Brennan R, Read RA, et al. Epidemiology of trauma deaths: a reassessment. J Trauma. 1995 Feb;38(2):185–93.

19. Cosgriff N, Moore EE, Sauaia A, Kenny-Moynihan M, Burch JM, Galloway B. Predicting life-threatening coagulopathy in the massively transfused trauma patient: hypothermia and acidoses revisited. J Trauma. 1997 May;42(5):857–61; discussion 861–2.

20. Hess JR, Brohi K, Dutton RP, Hauser CJ, Holcomb JB, Kluger Y, et al. The coagulopathy of trauma: a review of mechanisms. J Trauma. 2008 Oct;65(4):748–54.

21. Maegele M, Lefering R, Yucel N, Tjardes T, Rixen D, Paffrath T, et al. Early coagulopathy in multiple injury: an analysis from the German Trauma Registry on 8724 patients. Injury. 2007 Mar;38(3):298–304.

22. MacLeod JB a, Lynn M, McKenney MG, Cohn SM, Murtha M. Early coagulopathy predicts mortality in trauma. J Trauma. 2003 Jul;55(1):39–44.

23. Davenport R, Khan S. Management of major trauma haemorrhage: treatment priorities and controversies. Br J Haematol. 2011;155(5):537–48.

24. Brohi K, Singh J, Heron M, Coats T. Acute traumatic coagulopathy. J Trauma. 2003 Jun;54(6):1127–30.

25. Brohi K, Cohen MJ, Ganter MT, Matthay M a, Mackersie RC, Pittet J-F. Acute traumatic coagulopathy: initiated by hypoperfusion: modulated through the protein C pathway? Ann Surg. 2007 May;245(5):812–8.

26. Brohi K, Cohen MJ, Davenport R a. Acute coagulopathy of trauma: mechanism, identification and effect. Curr Opin Crit Care. 2007 Dec;13(6):680–5.

27. Gando S, Tedo I, Kubota M. Posttrauma coagulation and fibrinolysis. Crit Care Med. 1992 May;20(5):594–600.

28. van Hinsbergh VW, Bertina RM, van Wijngaarden A, van Tilburg NH, Emeis JJ, Haverkate F. Activated protein C decreases plasminogen activator-inhibitor activity in endothelial cell-conditioned medium. Blood. 1985 Feb;65(2):444–51.

29. Rezaie AR. Vitronectin Functions as a Cofactor for Rapid Inhibition of Activated Protein C by Plasminogen Activator Inhibitor-1. J Biol Chem. 2001;276(19):15567–70.

30. Brohi K, Cohen MJ, Ganter MT, Schultz MJ, Levi M, Mackersie RC, et al. Acute coagulopathy of trauma: hypoperfusion induces systemic anticoagulation and hyperfibrinolysis. J Trauma. 2008 May;64(5):1211–7; discussion 1217.

31. Frith D, Goslings JC, Gaarder C, Maegele M, Cohen MJ, Allard S, et al. Definition and drivers of acute traumatic coagulopathy: Clinical and experimental investigations. J

Thromb Haemost. 2010;8(9):1919–25.

32. Cohen MJ, Call M, Nelson M, Calfee CS, Esmon CT, Brohi K, et al. Critical role of activated protein C in early coagulopathy and later organ failure, infection and death in trauma patients. Ann Surg. 2012 Feb;255(2):379–85.

33. Henry D a, Carless P a, Moxey AJ, O'Connell D, Stokes BJ, McClelland B, et al. Anti-fibrinolytic use for minimising perioperative allogeneic blood transfusion. Cochrane Database Syst Rev. 2007;(4):1–3.

34. Shakur H, Roberts I, Bautista R, Caballero J, Coats T, Dewan Y, et al. Effects of tranexamic acid on death, vascular occlusive events, and blood transfusion in trauma patients with significant haemorrhage (CRASH-2): a randomised, placebo-controlled trial. Lancet. 2010 Jul 3;376(9734):23–32.

35. QUICK AJ, STANLEY-BROWN M, BANCROFT FW. A STUDY OF THE COAGULATION DEFECT IN HEMOPHILIA AND IN JAUNDICE.*. Am J Med Sci. 1935 Oct;190(4):501–10.

36. LANGDELL RD, WAGNER RH, BRINKHOUS KM. Effect of antihemophilic factor on one-stage clotting tests; a presumptive test for hemophilia and a simple one-stage antihemophilic factor assy procedure. J Lab Clin Med. 1953 Apr;41(4):637–47.

37. Davenport R, Manson J, De'Ath H, Platton S, Coates A, Allard S, et al. Functional definition and characterization of acute traumatic coagulopathy. Crit Care Med. 2011;(January 2016):1.

38. Schöchl H, Nienaber U, Hofer G, Voelckel W, Jambor C, Scharbert G, et al. Goal-directed coagulation management of major trauma patients using thromboelastometry (ROTEM)-guided administration of fibrinogen concentrate and prothrombin complex concentrate. Crit Care. 2010 Jan;14(2):R55.

39. HARTERT H. Die Thrombelastographie in der Differentialdiagnose der häAmorrhagischen Diathesen. Schweiz Med Wochenschr. 1949 Apr 9;79(14):318–22.

40. Dirkmann D, Görlinger K, Dusse F, Kottenberg E, Peters J. Early thromboelastometric variables reliably predict maximum clot firmness in patients undergoing cardiac surgery: A step towards earlier decision making. Acta Anaesthesiol Scand.

2013;57(5):594–603.

41. Huissoud C, Carrabin N, Audibert F, Levrat A, Massignon D, Berland M, et al. Bedside assessment of fibrinogen level in postpartum haemorrhage by thrombelastometry. BJOG An Int J Obstet Gynaecol. 2009;116(8):1097–102.

42. Dumitrescu G, Januszkiewicz A, Ågren A, Magnusson M, Isaksson B, Wernerman J. The temporal pattern of postoperative coagulation status in patients undergoing major liver surgery. Thromb Res. Elsevier B.V.; 2015;136(2):402–7.

43. Schöchl H, Frietsch T, Pavelka M, Jámbor C. Hyperfibrinolysis after major trauma: differential diagnosis of lysis patterns and prognostic value of thrombelastometry. J Trauma. 2009;67(1):125–31.

44. Holst J. Thrombelastography and rotational thromboelastometry early amplitudes in 182 trauma patients with clinical suspicion of severe injury: Meyer AS, Meyer MA, S??rensen AM, et al. J Trauma Acute Care Surg 2014;76:682-90. J Emerg Med. 2014;46(6):875–6.

45. Schochl H, Maegele M, Solomon C, Gorlinger K, Voelckel W. Early and individualized goal-directed therapy for trauma-induced coagulopathy. Scand J Trauma Resusc Emerg Med. BioMed Central Ltd; 2012 Feb 24;20(1):15.

46. Inaba K, Rizoli S, Veigas P V., Callum J, Davenport R, Hess J, et al. 2014 Consensus conference on viscoelastic test–based transfusion guidelines for early trauma resuscitation. J Trauma Acute Care Surg. 2015;78(6):1220–9.

47. Smith A a, Jacobson LJ, Miller BI, Hathaway WE, Manco-Johnson MJ. A new euglobulin clot lysis assay for global fibrinolysis. Thromb Res. 2003;112(5-6):329–37.

48. Katz J, Lurie A, Becker D, Metz J. The euglobulin lysis time test : An ineffectual monitor of the therapeutic inhibition of fibrinolysis. 1970;(December 1969):529–32.

49. Levrat a, Gros a, Rugeri L, Inaba K, Floccard B, Negrier C, et al. Evaluation of rotation thrombelastography for the diagnosis of hyperfibrinolysis in trauma patients. Br J Anaesth. 2008 Jun;100(6):792–7.

50. Rugeri L, Levrat a., David JS, Delecroix E, Floccard B, Gros a., et al. Diagnosis of early coagulation abnormalities in trauma patients by rotation thrombelastography. J Thromb Haemost. 2007;5:289–95.

51. Lang T, Bauters A, Braun SL, Pötzsch B, von Pape K-W, Kolde H-J, et al. Multi-centre investigation on reference ranges for ROTEM thromboelastometry. Blood Coagul Fibrinolysis. 2005;16(4):301–10.

52. Anderson L, Quasim I, Steven M, Moise SF, Shelley B, Schraag S, et al. Interoperator and intraoperator variability of whole blood coagulation assays: A comparison of thromboelastography and rotational thromboelastometry. J Cardiothorac Vasc Anesth. Elsevier; 2014;28(6):1550–7.

53. Baker, Susan P.Brian O'Neill. William Haddon . Willian B Long, Baker SP, O'Neill B, Haddon W, Long WB. The injury severity score: a method for describing patients with multiple injuries and evaluating emergency care. J Trauma. 1974 Mar;14(3):187–96.

54. Ganter MT, Hofer CK. Coagulation Monitoring: Current Techniques and Clinical Use of Viscoelastic Point-of-Care Coagulation Devices. Anesth Analg. 2008;106(5):1366–75.

55. von dem Borne PA, Cox LMP, Bouma BN. Factor XI enhances fibrin generation and inhibits fibrinolysis in a coagulation model initiated by surface-coated tissue factor. Blood Coagul Fibrinolysis. 2006 Jun;17(4):251–7.

56. Cesarman-Maus G, Hajjar K a. Molecular mechanisms of fibrinolysis. Br J Haematol. 2005 May;129(3):307–21.

57. Rijken DC, Lijnen HR. New insights into the molecular mechanisms of the fibrinolytic system. J Thromb Haemost. 2009;7(1):4–13.

58. Sakata Y, Loskutoff DJ, Gladson CL, Hekman CM, Griffin JH. Mechanism of protein C-dependent clot lysis: role of plasminogen activator inhibitor. Blood. 1986 Dec;68(6):1218–23.

59. Chapman MP, Moore EE, Moore HB, Gonzalez E, Gamboni F, Chandler JG, et al. Overwhelming tPA release, not PAI-1 degradation, is responsible for hyperfibrinolysis in severely injured trauma patients. J Trauma Acute Care Surg. 2015;80(1):1.

60. Bajzar L, Morser J, Nesheim M. TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin

complex. J Biol Chem. 1996 Jul 12;271(28):16603–8.

61. Sakharov D V., Plow EF, Rijken DC. On the mechanism of the antifibrinolytic activity of plasma carboxypeptidase B. J Biol Chem. 1997;272(22):14477–82.

62. Wang W, Boffa MB, Bajzar L, Walker JB, Nesheim ME. A Study of the Mechanism of Inhibition of Fibrinolysis by Activated Thrombin-activable Fibrionlysis Inhibitor. J Biol Chem. 1998;273(42):27176–81.

63. Refino CJ, DeGuzman L, Schmitt D, Smyth R, Jeet S, Lipari MT, et al. Consequences of inhibition of plasma carboxypeptidase B on in vivo thrombolysis, thrombosis and hemostasis. Fibrinolysis and Proteolysis. 2000;14(5):305–14.

64. Minnema MC, Friederich PW, Levi M, Von Dem Borne PAK, Mosnier LO, Meijers JCM, et al. Enhancement of rabbit jugular vein thrombolysis by neutralization of factor XI. In vivo evidence for a role of factor XI as an anti-fibrinolytic factor. J Clin Invest. 1998;101(1):10–4.

65. Nagashima M, Werner M, Wang M, Zhao L, Light DR, Pagila R, et al. An inhibitor of activated thrombin-activatable fibrinolysis inhibitor potentiates tissue-type plasminogen activator-induced thrombolysis in a rabbit jugular vein thrombolysis model. Thromb Res. 2000 May 15;98(4):333–42.

66. Hoffman M. Remodeling the blood coagulation cascade. J Thromb Thrombolysis. 2003;16(1-2):17–20.

67. Asakai R, Chung DW, Davie EW, Seligsohn U. Factor XI deficiency in Ashkenazi Jews in Israel. N Engl J Med. 1991 Jul 18;325(3):153–8.

68. Bolton-Maggs PHB. Factor XI deficiency--resolving the enigma? Hematology Am Soc Hematol Educ Program. 2009;97–105.

69. Carrieri C, Galasso R, Semeraro F, Ammollo CT, Semeraro N, Colucci M. The role of thrombin activatable fibrinolysis inhibitor and factor XI in platelet-mediated fibrinolysis resistance: a thromboelastographic study in whole blood. J Thromb Haemost. 2011 Jan;9(1):154–62.

70. Mosnier LO, Buijtenhuijs P, Marx PF, Meijers JCM, Bouma BN. Identification of thrombin activatable fibrinolysis inhibitor (TAFI) in human platelets. Blood. 2003;101(12):4844–6.

71. Marx PF, Dawson PE, Bouma BN, Meijers JCM. Plasmin-mediated activation and inactivation of thrombin-activatable fibrinolysis inhibitor. Biochemistry. 2002 May 28;41(21):6688–96.

72. Kravtsov D V, Matafonov A, Tucker EI, Sun M-F, Walsh PN, Gruber A, et al. Factor XI contributes to thrombin generation in the absence of factor XII. Blood. 2009 Jul 9;114(2):452–8.

73. von dem Borne P a, Meijers JC, Bouma BN. Feedback activation of factor XI by thrombin in plasma results in additional formation of thrombin that protects fibrin clots from fibrinolysis. Blood. 1995;86(8):3035–42.

74. Von dem Borne PA, Bajzar L, Meijers JC, Nesheim ME, Bouma BN. Thrombin-mediated activation of factor XI results in a thrombin-activatable fibrinolysis inhibitor-dependent inhibition of fibrinolysis. J Clin Invest. 1997 May 15;99(10):2323–7.

75. Comprehensive molecular portraits of human breast tumours. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2012 Oct 4;490(7418):61–70.

76. Curtis C, Shah SP, Chin S-F, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature. 2012 Jun 21;486(7403):346–52.

77. Shen R, Olshen AB, Ladanyi M. Integrative clustering of multiple genomic data types using a joint latent variable model with application to breast and lung cancer subtype analysis. Bioinformatics. 2009 Nov 15;25(22):2906–12.

78. Nagi S, Bhattacharyya DK, Kalita JK. Gene expression data clustering analysis: A survey. 2011 2nd Natl Conf Emerg Trends Appl Comput Sci. Ieee; 2011 Mar;1–12.

79. Xu R, Wunsch DC. Clustering algorithms in biomedical research: a review. IEEE Rev Biomed Eng. 2010 Jan;3:120–54.

80. Little RJ, D'Agostino R, Cohen ML, Dickersin K, Emerson SS, Farrar JT, et al. The prevention and treatment of missing data in clinical trials. N Engl J Med. 2012 Oct 4;367(14):1355–60.

81. Liao SG, Lin Y, Kang DD, Chandra D, Bon J, Kaminski N, et al. Missing value

imputation in high-dimensional phenomic data: imputable or not, and how? BMC Bioinformatics. 2014;15(1):346.

82. Milligan GW, Cooper MC. A study of standardization of variables in cluster analysis. J Classif. 1988;5(2):181–204.

83. D'haeseleer P. How does gene expression clustering work? Nat Biotechnol. 2005 Dec;23(12):1499–501.

84. Sibson R. SLINK: an optimally efficient algorithm for the single-link cluster method. The Computer Journal. 1973. p. 30–4.

85. Raza I, Khan S, Rourke C, Davenport R, Platton S, Carvalho R, et al. Acute traumatic coagulopathy has multiple important patterns of coagulation system derangement. Shock. 2013;40:34.

86. Mi Q, Constantine G, Ziraldo C, Solovyev A, Torres A, Namas R, et al. A dynamic view of trauma/hemorrhage-induced inflammation in mice: principal drivers and networks. PLoS One. 2011 Jan;6(5):e19424.

87. White NJ, Contaifer D, Martin EJ, Newton JC, Mohammed BM, Bostic JL, et al. Early hemostatic responses to trauma identified with hierarchical clustering analysis. J Thromb Haemost. 2015;13(6):978–88.

88. Harhangi BS, Kompanje EJO, Leebeek FWG, Maas AIR. Coagulation disorders after traumatic brain injury. Acta Neurochir (Wien). 2008 Feb;150(2):165–75; discussion 175.

89. Cauchie P, Cauchie C, Boudjeltia KZ, Carlier E, Deschepper N, Govaerts D, et al. Diagnosis and prognosis of overt disseminated intravascular coagulation in a general hospital -- meaning of the ISTH score system, fibrin monomers, and lipoprotein-C-reactive protein complex formation. Am J Hematol. 2006 Jun;81(6):414–9.

90. Kawasugi K, Wada H, Hatada T, Okamoto K, Uchiyama T, Kushimoto S, et al. Prospective evaluation of hemostatic abnormalities in overt DIC due to various underlying diseases. Thromb Res. Elsevier Ltd; 2011;128(2):186–90.

91. Gando S. Disseminated intravascular coagulation in trauma patients. Semin Thromb Hemost. 2001 Dec;27(6):585–92.

92. Gando S, Otomo Y. Local hemostasis, immunothrombosis, and systemic disseminated intravascular coagulation in trauma and traumatic shock. Crit Care. 2015;19(1):72.

93. Moore HB, Moore EE, Liras IN, Gonzalez E, Harvin JA, Holcomb JB, et al. Acute Fibrinolysis Shutdown after Injury Occurs Frequently and Increases Mortality: A Multicenter Evaluation of 2,540 Severely Injured Patients. J Am Coll Surg. American College of Surgeons; 2016;222(4):347–55.

94. Ramos CR, Moore EE, Manco-Johnson ML, Silliman CC, Chapman MC, Banerjee A. Diagnosis of Hyperfibrinolysis in Trauma Requires Further Validation. J Thromb Haemost. 2013;n/a – n/a.